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THE ROLE OF pH IN THE PERMEABILITY OF CHLORELLA TO 2,4-D[1,2]

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The effects of weak acids on the physiological and metabolic processes of plants have been of interest to investigators for many years. This interest is in large part due to the ubiquity of such compounds in biology and agriculture. Normal metabolic substrates, metabolic inhibitors, natural products of varied roles, and, from the practical standpoint, plant growth regulators, fungicides, and insecticides, all include representatives of the class termed weak organic acids. If weak bases are considered to behave in a similar manner, additional physiologically important compounds may be included. An essential feature of such studies is the effect of the external pH on the observed responses, which was early recognized to be a factor of considerable importance in investigations of this type (29).

The problem of the effects of pH on the activity of a compound in controlling plant function may be resolved into two parts: (a) the effect on penetration, and (b) the effect on the form which reacts within the cell. Most studies dealing with this subject fail to distinguish clearly between these two aspects of the problem, although some (10, 11, 16, 17, 18, 19, 20, 25) have measured uptake by plants either directly or indirectly. More often, however, the study has involved the measurement of a response of the organism to externally applied base or acid (1, 2, 3, 4, 5, 6, 7, 8, 11, 22, 24, 26, 27, 28). Such experiments have usually been interpreted on the basis of an effect of the external pH on the reactions of the compound within the cell, which in turn control the observed response. This usually involves the assumption that either ions or molecules are the reactive agents. Of at least equal importance is the question of which form penetrates the cell, since the steady-state equilibria produced at different hydrogen-ion concentrations largely control the concentration of reactive material regardless of its identity.

Interpretations of results obtained in this field have ranged from a purely anion effect (7, 22) to the conclusion that only uncharged particles such as molecules are able to penetrate the lipid barrier presented by cell membranes, with the ions penetrating only slowly or not at all (1, 6, 8, 9, 11, 15, 16, 18, 19, 29), or that the form which reacts with the appropriate enzymes or cell constituents to produce an effect on the plant is primarily the molecule (2, 4, 5, 6, 7, 23, 24). Other mechanisms have been suggested to explain effects, not on the basis of the form which penetrates, but rather in terms of the effect of external pH on the internal cell pH, thus controlling either the relative concentration of the reacting molecule or ion, or by changing the cell constituents themselves due to the amphoteric nature of the enzymes assumed to be involved (2, 4, 19, 26, 27, 28).

The fact that a lower concentration of undissociated molecules in the bathing medium is usually required to produce a standard effect at the high end of the physiological pH range than is needed at lower pH values has been used by Simon and Beevers (26, 27) in the development of a generalized expression for the action of weak organic acids and base. This involves a situation in which the concentration of the molecules of a weak acid needed to produce a standard response remains constant at pH values below the pK_a (i.e., when more than 50 % of the total concentration is in the form of molecules), shows a slight rise around the pK_a but decreases at higher pH values. These authors offer several possible explanations for this relationship. One is predicated on a rise in internal pH with the pH outside the cell, increasing the concentration of anions within the cell and thus supplementing the activity of the molecules present. Another suggests that competition of the anion with OH^- for entry into the cell reduces the amount of anions penetrating as the external pH rises, thus maintaining a constant level inside the cell. However, these workers reject the hypothesis that both molecules and anions can participate in the effects of a weak acid, with the activity or penetration of the anion fixed at a constant fraction of the activity of the molecule (6).

When experimental determinations are to be interpreted in terms of permeability they should not be based on measurement of such factors as inhibition of respiration, stem or root elongation, or other such indirect effects. Each reaction included between the actual entry of a particle and measurement of its effect may, in turn, be affected by the entering molecule, as well as by environmental changes such as the pH of the medium. Accordingly, we have attempted

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to reduce to a minimum the effects of post-entry reactions and to study in as nearly as possible an isolated form the penetration of carboxyl-C^{14}-labeled 2,4-dichlorophenoxyacetic acid (2,4-D*) into cells of Chlorella.

Materials and Methods

The plant material used in this study was *Chlorella pyrenoidosa* Chick. (Emerson's strain). The cells were grown in a glass tube under continuous illumination, as previously described (15). For use in these experiments a suitable volume of cell suspension was removed from the tube, and the absorbance of an aliquot was determined in a colorimeter at 435 mμ. The cell suspension was diluted or concentrated by centrifugation to obtain a suspension with a standard absorbance of 0.6. This suspension was then concentrated 16 times by centrifuging successive aliquots of the dilute suspension in the same tube. The packed cells were resuspended in a citrate buffer (0.08 M) of a pH appropriate to the particular experiment. One ml of this concentrated suspension of cells in buffer was transferred to 10-ml centrifuge tubes which were placed in a water bath at 25° C. Duplicate tubes were prepared for each of the conditions to be studied.

After a temperature equilibration of 15 minutes, 1 ml of a solution of twice the desired concentration of 2,4-D, with 10 % of the total in the form of carboxyl-C^{14} 2,4-D, was quickly pipetted into each tube and the tube swirled for mixing. The time of addition of the 2,4-D* was taken as zero time for the particular tube. At 2.5 minutes prior to the end of the desired exposure time the tubes were removed from the water bath and placed in a centrifuge which was run 30 seconds at 3000 rpm and braked to a stop. The supernatant liquid was poured off, and the pH determined immediately. The cells were washed by resuspending in 2 ml of redistilled water, centrifuging for 30 seconds, and pouring off the supernatant. At this point the desired time interval was complete. Room temperatures during these experiments were fairly constant within one or two degrees of 25° C.

To kill the cells and make the contents readily available for counting, 1 ml of butylamine was added to the packed cells after the last centrifugation. The butylamine extract and the cell residue were transferred quantitatively to cupped aluminum planchets, rinsed twice with 95 % ethanol, and dried on a spinner under an infra red heat lamp. The use of butylamine made it possible to prepare a sample for counting with a layer of dry cells and cell contents of fairly uniform thickness which could be counted with reproducible self-absorption of the radiation from the carbon.

The activity of the samples was determined with the use of a "Micro-Mil" end-window flow counter, which has a high sensitivity to weak beta radiation but at the same time permits counting large numbers of samples through the use of automatic sample-changing equipment. The activity determined for each sample was corrected for background radiation, self-absorption in the preparation (using a self-absorption curve constructed by the use of butylamine-treated Chlorella cells), and carry-over of active material on the walls of the test tube during the centrifuging and washing process. Conversion to quantity of 2,4-D was made by reference to a standard curve covering the range of activity encountered in the tissue samples.

Results and Discussion

Time-Course of 2,4-D Uptake: Since it was desired to eliminate as nearly as possible the effect of metabolic uptake or reactions of 2,4-D with cell constituents, the time-course of uptake by Chlorella was determined. For this purpose duplicate aliquots of concentrated cell suspension in 0.04 M citrate buffer at pH 4.5 were allowed to take up 2,4-D* from solutions of 1×10^{-3} M and 5×10^{-5} M for intervals ranging from 2.5 to 80 minutes. The mean results of two experiments of this type are summarized in the two upper lines of figure 1.

It may be seen that at a concentration of 1×10^{-3} M there is a rapid initial uptake of 2,4-D*, which levels off in 10 to 20 minutes and is followed by a slower accumulation of 2,4-D* within the cells, which continues at a steady rate for at least 80 minutes. The more dilute solution results in less actual uptake, but if the values obtained are multiplied by 20 to put the two solutions on an equal concentration basis, the cells in 5×10^{-5} M 2,4-D* are found to exhibit an identical rate of uptake for periods up to 20 minutes indicating that uptake during this period is a linear function of concentration. The subsequent increase in activity within the cells has a relatively steeper slope here than in the more concentrated solution.

If the uptake from 0 to 20 minutes is attributed mainly to establishment of a diffusion equilibrium between the solution and the cells, then it seems likely that the subsequent slower rate of uptake is metabolic in nature and results from the reaction of 2,4-D with cell constituents, removing it from the diffusion equilibrium previously established. The different slopes for this part of the two curves could be attributed to enzyme saturation at the higher concentration and indicate that this second phase of the uptake follows an adsorption isotherm.

A similar series of time-uptake studies was carried out with the same concentrations of 2,4-D* at pH 7.0. In this case the absolute amount taken up by the cells was quite low relative to that at pH 4.5, but when these values were corrected for the differences in the concentration of undissociated molecules at the two hydrogen-ion concentrations, the values obtained were more than 10 times as great as those obtained at pH 4.5. The shape of the curve was essentially identical, however, with an initial steep rise, leveling off after 20 minutes and followed by a slower increase up to 80 minutes. Accordingly, it was decided to use 10 minutes as a standard time interval for further studies, since over a fairly wide range of concentration and pH this seemed to limit the cells to a condition

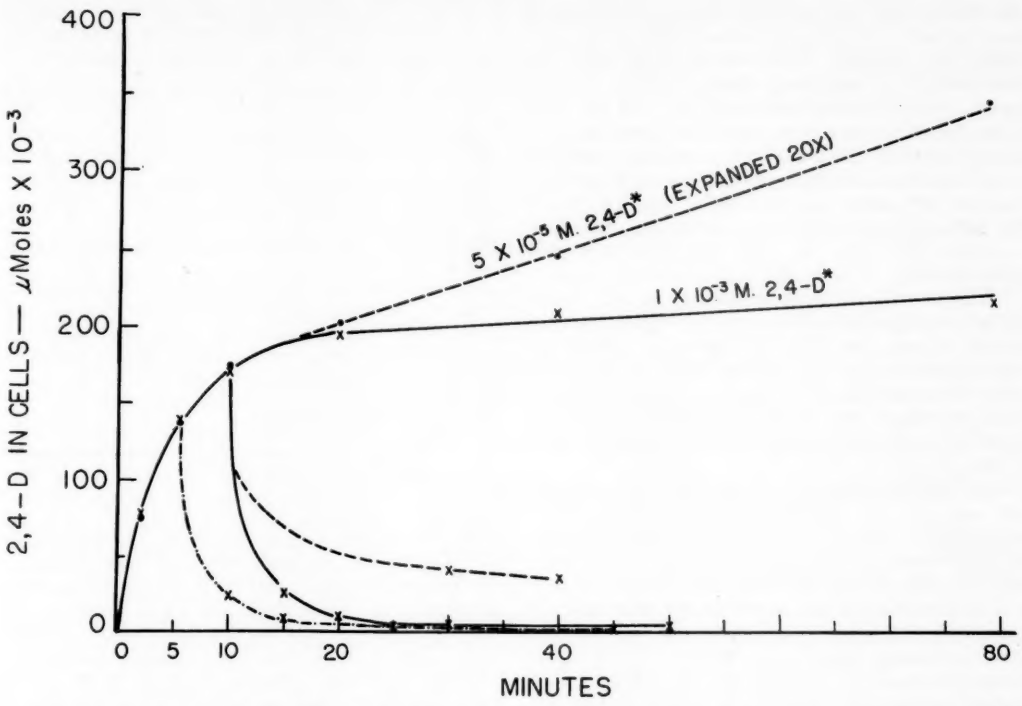

FIG. 1. Uptake of carboxyl-C^{14}-labeled 2,4-D* by Chlorella as a function of time (two upper lines), and loss to buffer containing unlabeled 2,4-D after 5 and 10 min exposure to 1×10^{-3} M 2,4-D* (two lower lines). All experiments with 0.04 M potassium citrate buffer at pH 4.5. Intermediate line represents loss to buffer containing no 2,4-D.

in which the uptake resulted mainly from a diffusion equilibrium established through the semipermeable membrane of the cells.

The fact that living cells were essential for this uptake was established by determining the uptake of 2,4-D* by dead Chlorella cells (heated to 90° C for 5 minutes). The 2,4-D* found in these cells was uniformly about 2 % of the amount found with living cells under the same conditions, and this probably represents adsorption to cell walls, denatured proteins, and other cell constituents.

The largely physical nature of the uptake being studied here is indicated by the ease with which the 2,4-D* taken up by the cells is lost either to a buffer containing no 2,4-D or one containing 1×10^{-3} M nonradioactive 2,4-D. When Chlorella cells are centrifuged out of a buffer containing labeled 2,4-D* and resuspended in buffer alone or buffer with added nonradioactive 2,4-D, the amounts of activity found remaining in the cells after a short time are indicated by the three lower lines of figure 1. It may be seen that 2,4-D* is readily stripped from the cells by inert 2,4-D, with most of the labeled material being removed within 5 minutes, with a gradual continuing elimination of the active material in the cells for periods up to 40 minutes. The amount remaining in the cells at this time, approximately 1.5 % of the initial maximum, probably represents the results of metabolic activity. It will be noted, however, that a considerable fraction of the original activity is not removed when the cells are placed in a buffer which does not contain 2,4-D. At pH 4.5 this adsorbed portion of the labeled compound consists of approximately 20 % of the initial (10-minute) maximum 30 minutes after the cells were transferred to non-labeled solutions. The initial phase of 2,4-D* uptake is thus shown to be comprised of both a reversible diffusion into the cell and a reversible adsorption within the cell. This is in agreement with the results of Johnson and Bonner (20) for the uptake of 2,4-D* by Avena coleoptile sections.

Further evidence for the non-metabolic nature of the process being studied here is offered by the fact that 1×10^{-2} M KCN added to the buffer did not result in a significant decrease in the uptake by the cells over a 10- or 20-minute interval.

EFFECT OF pH ON 2,4-D* UPTAKE OVER SHORT TIME INTERVALS: As has been pointed out earlier (4, 15), if the amount of undissociated molecules required to produce a standard response over a range of external hydrogen-ion concentrations is determined and the resulting concentrations are plotted against

the external pH, a straight line with zero slope should result if only the molecular form is effective in producing the response. This type of assay offers the best method of comparing relative effects of compounds under different conditions (3) and has been made for a variety of responses of plants to weak organic acids (4, 15, 26, 27, 28). In most cases the results have been of the type discussed by Simon and Beevers (27), where, at pH's higher than the pK of the acid involved, the line slopes downward, indicating a greater effect from the same amount of undissociated molecules.

When uptake is being studied as in our experiments, a standard effect cannot be determined, and an exactly comparable plot is impossible. However, when Chlorella cells are allowed to take up 2,4-D* from solutions of identical total concentration over a wide pH range, the results are similar to those in which a standard response has been determined over a similar range of pH.

In these experiments aliquots of a concentrated cell suspension in 0.04 M potassium citrate buffers ranging from pH 4.0 to pH 7.5 were allowed to take up 2,4-D* from a solution of 1×10^{-3} M for 10-minute intervals, and the amount which had entered the cells was determined. In this case if the molecule were the only form which could penetrate into the cell, the ratio of the total 2,4-D* inside the cell (T_1/V) to undissociated molecules outside the cell (HA_o), when plotted against the external pH (pH_o), should also yield a straight line with zero slope. A plot of this type is shown in figure 2 (20 determinations per point; final pH values averaged as hydrogen-ion concentrations). Here it may be seen that with an increase in pH_o, the ratio $\frac{T_1/V}{HA_o}$ increases; that is, more 2,4-D* enters the cell relative to the concentration of undissociated molecules in the bathing medium. From pH 5 to pH 7 this increase is about a threefold change per unit pH change. This increase in uptake of 2,4-D* relative to its molecular concentration, would correspond to the decrease in concentration of undissociated molecules required to produce a standard response in the studies mentioned above. Thus a relation of the same type as that discussed by Simon and Beevers (26, 27) for effects of weak acids on plant response is demonstrated at pH's above the pK of the acid for uptake of 2,4-D* under conditions in which the metabolic reaction is negligible. This failure of the amount of 2,4-D* penetrating the cells to be a linear function of the external molecule concentration indicates that ions must also be a factor in permeation at higher pH, or that some additional factors related to pH must also be involved. Some of the possible effects are discussed below.

Effect of Undissociated Buffer Acids: A change in the pH of the buffer system containing the acid results in a similar change in the ionization of the acid which composes the buffer system, as well as an increase in the ionization of the acid whose uptake is being measured. At pH 7 a buffer composed of 0.04 M citric acid will contain only 1/1000th as much undissociated citric acid as the same buffer at pH 4 (using only the pK of the first hydrogen). On this basis it seemed possible that the greater apparent uptake of 2,4-D relative to the amount of molecules present at pH 7 might actually be due to a decrease in the amount taken up at lower pH's as a result of increasing competition from the undissociated citric acid present at the lower pH. This could be a competition for binding sites on a carrier system as has been suggested by Epstein et al (12, 13, 14, 21) for uptake of inorganic anions and cations. However, it seems more likely that the interference is a simple noncompetitive reduction of the net diffusion of one solute by the presence of another (Raoult's Law). An effect of this type is also suggested by work done in our laboratory, in which the effect of acetic acid in citrate buffer on the photosynthesis of Chlorella is apparently related to changes in the amount of undissociated citric acid present over the same range of pH as was used in the present studies.

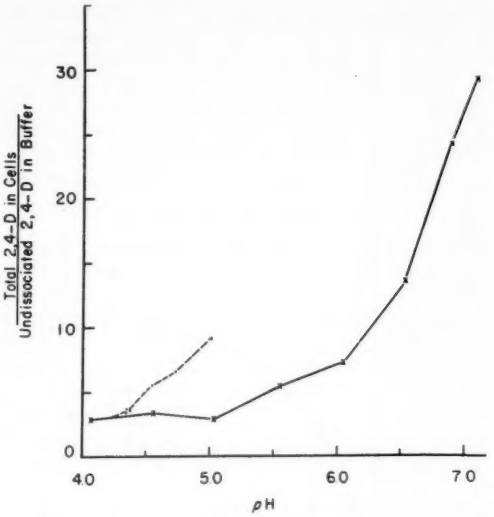

FIG. 2. Ratio of total amount of 2,4-D* taken up to concentration of undissociated 2,4-D* molecules in 0.04 M potassium citrate buffers of various hydrogen-ion concentrations. Solid line, total 2,4-D* taken up by Chlorella cells (T_1/V)/ undissociated 2,4-D* in buffer (HA_o), over the range from pH 4 to pH 7. Dashed line, (T_1/V)/(HA_o) from buffers at an intial pH of 4.0 with undissociated citric acid concentrations corresponding to 0.04 M potassium citrate buffers from pH 4 to pH 7.5.

To test this hypothesis a series of experiments was set up in which the initial pH of the preparations was established at 4.0, but in which individual treatments consisted of buffers containing amounts of undissociated citric acid comparable to those found in a 0.04 M buffer at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5; namely, 288, 94, 28.8, 9.4, 2.88, 0.94, 0.288, and 0.094 M $\times 10^{-5}$, respectively. The more dilute solutions were,

of course, poor buffers, and in these tubes the final pH was found to have drifted to approximately 5.0.

The effect of differences in pH can to some extent be eliminated by plotting $\frac{T_i/V}{HA_o}$ for these experiments against pH_o. This should produce a straight vertical line if no change occurred in the buffer pH, but experimentally it is found to slope upward from pH 4.0 to pH 5.0, as shown by the dashed line in figure 2. If the entire effect in increasing the ratio $\left[\frac{T_i/V}{HA_o}\right]$ with increasing pH were due to the release of a competition for entry by reducing undissociated citric acid in the buffer, the point corresponding to a buffer with an initial pH of 7.5 should rise to the same value on the Y axis as the value obtained with a buffer of that pH. Comparison of the two lines of figure 2 reveals that this is not the case. Although there appears to be some effect of the decrease in undissociated citric acid concentration in allowing more 2,4-D* to enter the cells, relative to the undissociated molecular concentration, its effect is shown mainly by those citric acid concentrations corresponding to buffers with pH's from 6.0 to 7.5, and the magnitude of the overall effect is not more than could be attributed to an increase of one unit in the buffer pH. It is therefore apparent that, although part of the effect noted in increasing 2,4-D* uptake with an increase in pH may be due to the accompanying change in undissociated buffer acid concentration, this will not account for the entire effect noted in these experiments.

Model Systems Employing Different Ion Permeabilities: Another approach to the problem of increased uptake relative to the undissociated molecule concentration at higher pH's is the suggestion of Brian (6) that both molecules and ions may be effective, but that the ions contribute only $1/n$th of the total activity. Simon and Beevers (25) have criticized this suggestion and state that "no single value of n will fit observations which cover a wide pH range." The range referred to in their example is from pH 2 to pH 8. Since pH values below 4 appear to be injurious to our Chlorella, and since citrate buffers will not maintain a pH much above 7 in the presence of Chlorella, we have not been able to cover this entire range but do have determinations for a range from pH 4 to pH 7. Accordingly, an attempt has been made to evaluate our experimental data in terms of a permeability to ions which is a constant fraction of the permeability to molecules.

If the assumption is made that our data represent purely a diffusion through a semipermeable membrane, the equilibrium situation for the weak acid being measured (neglecting the buffer acid) can be represented in the following manner:

$$Q\,HA_o + A^-_o = Q\,HA_i + A^-_i \qquad (1)$$

Where: HA_o = concentration of molecules external to the cell.

A^-_o = concentration of anions external to the cell.

HA_i = concentration of molecules within the cell.

A^-_i = concentration of anions within the cell.

Q = permeability of cell to molecules/permeability to anions.

Equation *1* may be made applicable to specific situations by expressing the various concentrations in terms which may be experimentally determined:

$$\frac{QT_o}{R_o} + T_o - \frac{T_o}{R_o} = \frac{QT_i}{R_i} + T_i - \frac{T_i}{R_i} \qquad (2)$$

Where: T_o = total concentration of acid in buffer (micromoles/liter).

R_o = 1 + antilog ($pH_o - pK$), pH_o being the pH of the buffer at the end of the uptake period. T_o/R_o thus becomes the concentration of molecules in the buffer at pH_o[3] and $T_o - T_o/R_o$ would be equal to the anion concentration at pH_o.

T_i = total concentration of acid in the cells (micromoles/liter).

R_i = 1 + antilog ($pH_i - pK$). T_i/R_i thus represents the molecule concentration at the pH of the cell and $T_i - T_i/R_i$ is the anion concentration at pH_i.

This expression states that the molecules moving into the cell, plus the anions moving into the cell, equal the molecules moving out of the cell, plus the anions moving out of the cell, and is based on the following assumptions:

1. Particles can move with equal facility in either direction through the semipermeable membrane of the cell.

2. The rate of movement in each direction will be proportional to the respective concentrations.

3. The relative proportions of molecules and anions will be determined by the pH of the buffer or of the cell. As an initial premise it is assumed that the buffering systems both inside and outside the cell will resist changes in pH resulting from movement of 2,4-D.

4. The time constant for the molecule $\rightleftarrows$ ion conversion is short relative to that for movement of particles across the membrane.

5. The volume of the external medium is infinitely large with respect to the volume of the cells, so that no change in external concentration will occur as a result of 2,4-D movement into the cells.

By substituting different values of Q in equation *2* and solving for T_i we can produce a family of curves such as are shown in figure 3. Here T_i has been expressed as micromoles by dividing concentration by volume of the cells (T_i/V).

[3] This relationship derives from the mass action expression for ionization of the acid involved: [HA] = [T]/1 + antilog (pH − pK), [HA] being concentration of unionized molecules and [T] the total concentration of the acid. For these studies the pK of 2,4-D was taken as 2.96 (15).

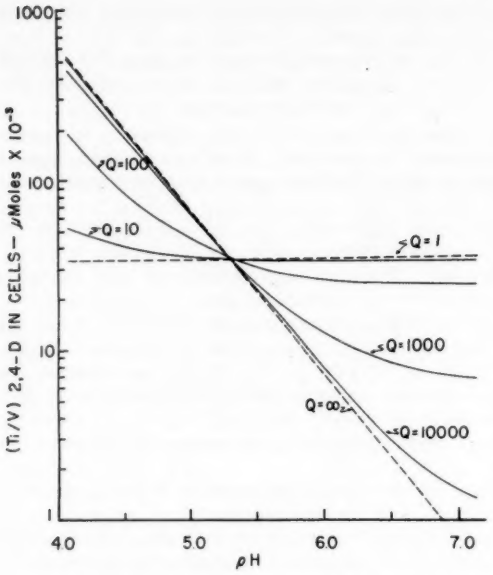

FIG. 3. Uptake of weak acids from buffers of various hydrogen-ion concentrations predicted by a model system based on permeability to both molecules and ions.

When Q = 1 (the cell equally permeable to both ions and molecules), the expression reduces to:

$$T_i = T_o \qquad (3)$$

If we use this with experimental values which include a V of 0.0333 ml determined by centrifuging cells into capillary tubes, and a concentration of 1×10^{-3} M 2,4-D, a horizontal line is produced (Q = 1, fig 3) with a value of 33.3 micromoles $\times 10^{-3}$ (33.3×10^{-6} liters $\times 1^3$ micromoles/liter).

If we go to the other extreme and make Q = ∞ (the cell permeable only to molecules), the expression reduces to:

$$T_i = T_o\left(\frac{R_i}{R_o}\right) \qquad (4)$$

This produces a straight line if T_i/V is plotted on a log scale against pH_o. The intersection of this line with the Q = 1 line would be at a pH_o value which is equal to pH_i (since, then, $R_i/R_o = 1$), and in figure 3 this value has been established as 5.30 for reasons developed below. As has already been brought out, this expression for permeation exclusively by molecules does not account for the experimental data. However, if we make Q = some value > 1 but < ∞, we produce the solid lines shown in figure 3. When Q is small the line is pulled up rapidly at pH's below pH_i, but approaches the Q = 1 situation at higher pH's. When Q becomes large, the situation is reversed, with T_i/V values which approach Q = ∞ at low pH's, but tend to pull away from this line with an increase in pH_o.

When Q is made a variable with a value greater than 1 but less than ∞, equation *2* reduces to:

$$T_i = \frac{R_i T_o}{Q + R_i - 1} + \frac{R_i T_o (Q-1)}{Q + R_i - 1}\left(\frac{1}{R_o}\right) \qquad (5)$$

which is of the type Y = A + BX, and which may be used to fit the experimental values for T_i to the pH_o values by the method of least squares. In this expression Q = 1 + B/A and, knowing Q, we may calculate pH_i from R_i.

The results of fitting the experimental data for amounts of 2,4-D* inside the cells of Chlorella at pH's from 4 to 7 with 1/1 + antilog ($pH_o - pK$) are shown in figure 4, where the calculated line is shown as log T_i/V plotted against pH_o. The equation which produces this line is found to be $T_i/V = 5.46 + 6069$ [1/1 + antilog ($pH_o - pK$)], and this makes Q = 1112 and pH_i 5.30. The fit of this line is mathematically excellent with r = 0.997, but when plotted in the manner used in figure 4 it is obvious that the calculated line predicts a slightly greater uptake of 2,4-D* than was actually found at pH's above 6.0. If the effect of decreasing the undissociated citric acid concentration at the higher pH's, discussed earlier, is subtracted from the experimental values, the value of Q is increased to 1258 and the calculated line comes nearer the experimental points between pH 6 and pH 7, but the general shape of the curve is unchanged.

Another factor which will affect the actual amount of 2,4-D* which is available within the cell for a dif-

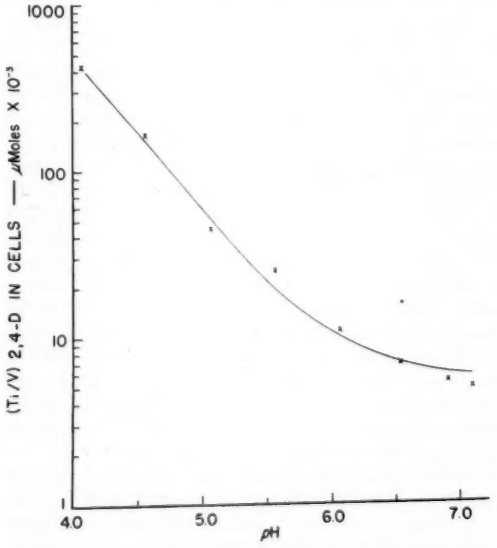

FIG. 4. Amount of 2,4-D* found in Chlorella cells as a function of the external pH over the range from pH 4 to pH 7. The x's represent mean values for 20 experimental determinations. The line is fitted by the model system of figure 3 with Q fixed at a value greater than 1 but less than infinity. The fitting process indicates a value of 1112 for Q and a cell pH of 5.30.

fusion equilibrium is the amount of the 2,4 D* which is adsorbed after entering the cell and thus removed from the equilibrium. The amount of adsorbed material was determined by allowing cells to take up labeled 2,4-D from buffers at different pH for a 10-minute interval. A portion of the cells at each pH value were centrifuged out and resuspended in citrate buffer at the same pH containing no 2,4-D. A second portion was centrifuged and resuspended in buffer of the same pH containing non-radioactive 2,4-D. A third lot of cells was centrifuged, washed and killed immediately to serve as an uptake control. The cells suspended in buffer alone and buffer plus 2,4-D were incubated an additional 30 minutes, at which time they too were killed. The amount of 2,4-D* found in each group of the cells at each pH was used to determine the percentage of total uptake at that pH which was adsorbed as indicated by the difference between the loss to buffer alone and buffer with 2,4-D. The results of these experiments over the range from pH 4.0 to 7.0 are summarized in figure 5. The percentage of the total 2,4-D* taken up in 10 minutes which is adsorbed is plotted against the pH and shows a decreasing percentage with an increase in pH. The equation for the line is $Y = 57.6 - 8.3\,X$ with a correlation coefficient $r = -0.921$.

This relationship of a decreasing percentage of bound 2,4-D* with an increase in pH appears to indicate either that a large proportion of the total bound 2,4-D* is adsorbed on the outer surface of the cell where charged binding sites could readily be affected by the external buffer, or that internal cell pH is being appreciably changed in response to changes in buffer pH. Alternatively, it might be possible that only the undissociated molecules are adsorbed.

The effect of this differential binding of 2,4-D* on the fit of the model system to the experimental data was tested by subtracting from the experimental T_i/V values the percentage of bound 2,4-D* plus the effect of undissociated citric acid at each pH before performing the computation indicated in equation *5*. This procedure resulted in a line with the equation $T_i/V = 5.54 + 4624\ [1/1 + \text{antilog}\,(pH_o - pK)]$, which makes $Q = 835$ and indicates an internal pH of 5.78. This decrease in Q and increase in pH_i results from the inverse relationship of adsorbed 2,4-D* to the $\frac{T_i/V}{HA_o}$ change predicted by the model system. Since the adsorbed acid cannot participate in a diffusion equilibrium, these figures probably more nearly approximate the actual pH of the cells and the relative permeability of Chlorella to 2,4-D molecules and ions.

It is apparent that by assuming a value for the permeability of the cell to ions which is $1/n$th the value of the permeability to molecules, and a constant internal pH, it is possible to produce a mathematical expression which will fit data similar to those obtained (27) for the change in the effect of a weak acid with an increase in the pH of the bathing medium. The fact that a model system will describe a relationship of the type found experimentally for the uptake of

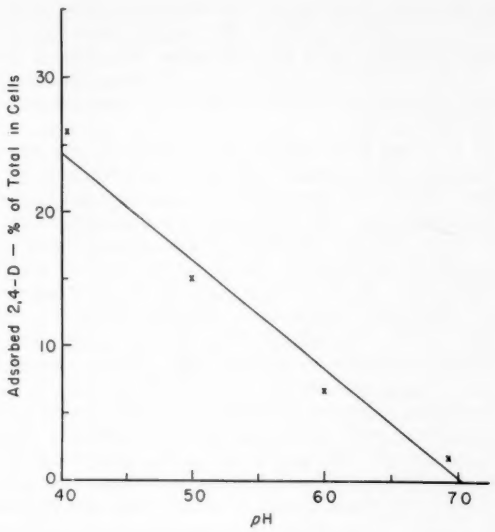

FIG. 5. 2,4-D* adsorbed in Chlorella cells after 10 minutes uptake and 30 minutes leaching as a function of external pH over the range from pH 4 to pH 7. The fraction of the total 2,4-D* which is bound by the cells is determined by leaching one set of cells with buffer alone and a second set with buffer plus inert 2,4-D at each pH.

2,4-D* by Chlorella does not indicate that this is the manner in which the experimental data have been produced. Other factors, individually or in combination, could also result in the same experimental findings.

Effect of Changes in the Internal pH of the Cell: One factor which could bring about results of the type found in these studies, and which has frequently been suggested in this connection, is a change in the cell pH resulting from and related to the pH of the buffer in which the cells are bathed. The possibility of changing the cell pH by immersion in a buffer having a pH different from that of the cell has been widely discussed. Experimental data have been presented to show both that this change does not occur (1, 9, 11, 17, 29) and that it occurs readily (9, 11, 18, 19, 29).

Accurate measurement of internal pH is difficult and, with an organism of the Chlorella type, almost impossible. If the assumption is made that the resistance of cells to internal pH changes is primarily due to the impermeability of the cell to H^+ and OH^- ions, but that undissociated molecules can enter readily, the question of whether a given buffer will change the pH of a cell should depend on the dissociation characteristics of the acid and salt which compose the buffer. If the acid and its salt are weakly dissociated they should be able to penetrate the cell, and on entering should tend to bring the pH of the cell toward the value for the acid or salt concerned. Under these conditions the pH of a cell in a buffer of

a pH lower than that of the cell should tend to decrease somewhat owing to the entry of undissociated acid molecules, while at a pH higher than that of the cell, the entry of undissociated salt molecules would provide a supply of cations and should tend to raise the internal pH.

It is well known that cations such as K^+ can readily enter cells, possibly making use of a charge-reducing "carrier" for the purpose of passing through the membrane (13, 14). Nielson and Overstreet (23), working with an unbuffered system, have found that less K^+ is absorbed by barley roots at lower pH's, which they interpret as a direct effect of H^+ on the K^+ absorption mechanism. This K^+ uptake would also tend to raise cell pH when a large supply of the cations is present in the bathing medium. Johnson and Bonner (20) showed that 2,4-D* was taken up by Avena coleoptile more rapidly in the presence of potassium salts including KCl, K maleate and KH_2PO_4, with the salts of the weaker acids being the more effective. These workers also found that Na, Ca and Mg could replace K in increasing the uptake of 2,4-D*. These results may be due to an increase in the cell pH since the effect of raising the pH in this fashion would be to increase the total uptake. Citric acid has been stated (29) to penetrate plants only with difficulty, but for the purpose of discussion the potassium citrate buffer used in these experiments will be assumed to perform with Chlorella in the manner described above.

Returning to the model system discussed earlier, if we assume that ions cannot penetrate the cell ($Q = \infty$), then it follows that any experimental change from the straight line $Q = \infty$, shown in figure 3, may be attributed to an internal pH change, since when $Q = \infty$, $T_i = T_o$ (R_i/R_o), under these conditions, using the model system and T_i expressed as micromoles, one can derive the relationship:

$$pH_i = \log\left[\left(\frac{T_i R_o}{T_o V}\right) - 1\right] + pK \qquad (6)$$

which will give the internal pH required to alter the relative amounts of molecules and ions within the cell in equilibrium with the molecular concentration in the buffer so as to provide a total amount of 2,4-D* within the cells equal to that which was actually found. The results of substituting these calculated values of pH_i into the R_i component of the equation for the $Q = \infty$ condition are shown in the fitted line of figure 6.

As a means of demonstrating the fact that pH changes in the cells might occur and could account for at least some of the observed responses of Chlorella, consideration was given to the possibility that a buffer composed of citric acid with a weakly basic cation might, even though it readily penetrated the cells, have less tendency to raise the cell pH than the potassium citrate buffer used in the other experiments reported here. Accordingly, a series of experiments was run in which the uptake of 2,4-D* from potassium citrate and ammonium citrate buffers of equal citrate concentration was determined. These buffers ranged from pH 4.0 to pH 7.0. A summary of these experiments is given in figure 7, where $\frac{T_i/V}{HA_o}$ is plotted against pH_o for the two buffer systems. It may be seen that the tendency is for the Chlorella to take up more 2,4-D*, relative to the undissociated molecule concentration, from a potassium citrate buffer than from an ammonium citrate buffer, thus appearing to confirm the hypothesis stated above. However, the systems involved are of such complexity that generalizations based on data of this type seem undesirable.

Conclusions

In the experiments reported, measurements have been made of the effect of a change in the pH of an external buffer on the uptake of labeled 2,4-D* by cells of Chlorella over short intervals of time. The time involved and other considerations indicate that the uptake measured is mainly a diffusion equilibrium through the semipermeable membrane of the living cell and is non-metabolic in nature. A portion of the total is removed from the diffusion equilibrium by adsorption within or on the cell. These experiments have resulted in data which are similar to those frequently reported for an influence of pH on the activity of various weak organic acids in bringing about changes

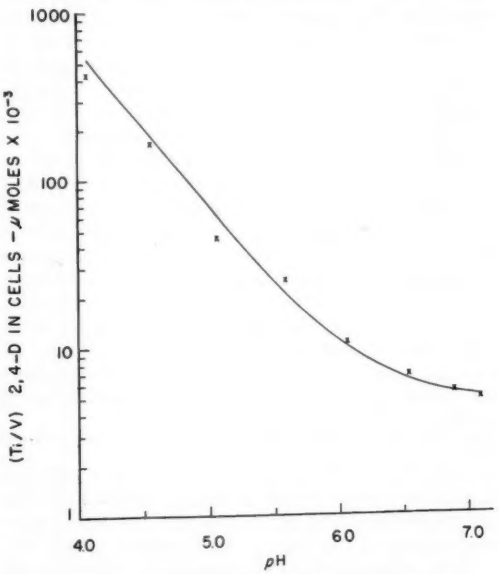

Fig. 6. Amount of 2,4-D* found in Chlorella cells as a function of the external pH over the range from pH 4 to pH 7. The x's are the same experimental values used in figure 4. The line is fitted by the model system of figure 3 with the assumption that the cell is impermeable to ions and that deviations from the linear $Q = \infty$ condition are due to changes in cell pH.

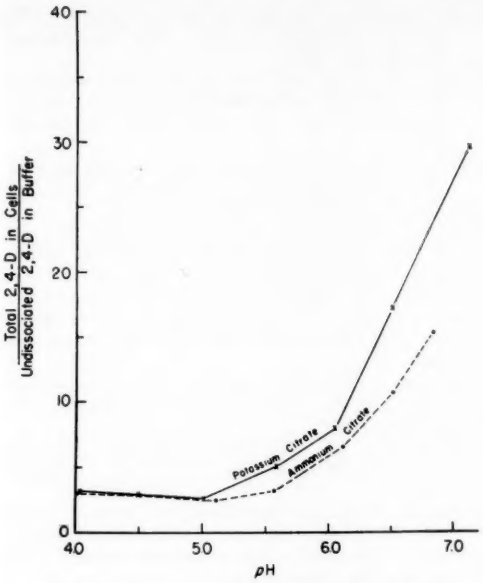

FIG. 7. Ratio of total amount of 2,4-D* taken up by Chlorella to concentration of undissociated 2,4-D* molecules in 0.04 M potassium citrate buffers and 0.04 M ammonium citrate buffers over a range from pH 4 to pH 7.

in the function of plant cells. That is, our data also show the progressive increase in uptake (or effect), relative to the concentration of undissociated molecules, as the external pH rises above the pK of the acid. Since we find that uptake is affected by external pH in the same manner as is the "activity" of an acid measured in longer-term experiments, and since penetration into the cell must precede any metabolic reaction occurring within the cell, it does not seem unreasonable to assume that the influence of pH on weak acid effects on plants is exerted primarily through its control of the diffusion equilibrium established between the buffer and the cells. If this is the case, the question of which form of a weak acid participates in reactions within a cell is of secondary importance in discussions of weak acid–pH interactions, since the diffusion equilibrium will control the concentration of the reactant.

The data presented here seem susceptible to three or more interpretations with respect to the amount of acid found within cells, relative to the molecular concentration in buffers of various hydrogen-ion concentrations.

1. One possibility is that the cells will take up molecular 2,4-D* with relative ease, and that the anion will also penetrate the cell, but at a rate considerably slower than the uncharged particle. A model system based on the assumptions of an unchanging internal pH and penetration of ions at a fixed ratio to the molecules can be shown mathematically to fit the data of these experiments. The calculated penetrability of 2,4-D* anions is found to be about 1/1000th of that of the molecules when the data are fitted on the basis of this model system.

2. Another possibility is that the cells are truly impermeable to anions, but that the pH's of the cells are changed by contact with buffers having pH's differing from those of the cells. For the present results, dealing with a range of external pH's from 4.0 to 7.0, the change in internal pH required to produce the observed rates of uptake would be a total of 0.8 pH. If the initial pH of the cells is assumed to be 5.30, the required change would be from pH 5.22 to pH 6.01.

3. An interference of the undissociated molecules of the buffer acid with the uptake of 2,4-D* is also a possible explanation. Since the concentration of undissociated molecules in the buffer decreases as the pH of the buffer rises, the greater uptake at high pH would be due to a release of the uptake-inhibiting interference found at lower pH's. It can be shown that such an effect does exist, but under the present conditions its magnitude is insufficient to account for the entire observed effect.

It seems likely that a combination of the third possibility, the effect of which can be measured, with one or both of the other mechanisms, which cannot be measured with any precision, is the method by which the observed deviations from strict proportionality with undissociated molecule concentrations are brought about.

SUMMARY

1. The uptake of carboxyl-C^{14} 2,4-D by Chlorella has been studied as a function of the pH of buffers in which the cells are suspended.

2. Within periods of 10 minutes the uptake is found to consist primarily of diffusion into the cell and adsorption within the cell. This initial uptake is followed by a slower, "metabolic" uptake.

3. The amount of 2,4-D* taken up by the cells relative to the undissociated molecule concentration in the buffer is found to increase with an increase in pH.

4. A model system which assumes the permeability of the cell to 2,4-D* anions to be a constant fraction of its permeability to 2,4-D* molecules was developed to express the effect of pH on the diffusion equilibrium between the external buffer and the cells. This model is found to fit the experimental data well on the assumption of either a constant internal pH of the cell, or a change in cell pH with that of the buffer. When a constant cell pH is assumed, the fitting process indicates that the permeability of Chlorella cells to 2,4-D molecules is 800 to 1000 times as great as the permeability to anions, and that the cell pH is 5.3 to 5.8.

The authors gratefully acknowledge their indebtedness to Mr. L. E. Brown, Miss Kay Black and Mr. B. L. Brannaman for stimulating discussion and technical assistance.

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ON THE UPTAKE OF CARBON DIOXIDE AND BICARBONATE BY ROOTS, AND ITS INFLUENCE ON GROWTH[1]

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From the viewpoint of the efficient use of light in photosynthesis, algae possess the advantage (over higher plants) that the medium in which they grow can be readily enriched with bicarbonate, and growth correspondingly increased. For higher plants, unfortunately, it would hardly be possible to enrich the atmosphere appreciably with CO_2. However, it occurred to one of us that it might be practical to enrich the soil or the nutrient solution (17). One or two unsuccesful attempts at this have been made in the past, but without any systematic study of the factors involved.[4] Recent claims of increased yields following carbonate fertilization, by Kursanov and coworkers in Russia (see below) make a study of this problem more urgent. Such a practice would depend critically on whether roots can absorb CO_2 or bicarbonate to an appreciable extent. The present work was therefore undertaken to determine the amount of CO_2 or bicarbonate which could be absorbed by the roots of intact plants, and to study the effect of CO_2, applied in the root medium, on the growth of the root system and of the whole plant. Both monocotyledons and dictyledons have been included in the experiments.

It is known, of course, that roots, like other non-green tissues, are capable of fixing CO_2. Ruben and Kamen (14) demonstrated uptake of $C^{11}O_2$ by a preparation of ground barley roots as long ago as 1940, but the short half-life of the isotope used prevented identification of the compounds into which the CO_2 was incorporated. Overstreet, Ruben and Broyer studied the uptake of bicarbonate ions by excised barley roots over a short period (11). Their data allow an approximate comparison of the amounts taken up with the amount produced by respiration; with their "low-salt" plants, using $KHC^{13}O_3$ as bicarbonate source, it develops that the uptake represented about 10 % of respiration. However, the amount of K^+ taken up was three to six times as large as the amount of HCO_3^- taken up in the same length of time. More recently, Poel (12) has repeated and extended these experiments, using C^{14} and radiochromatographic techniques, and has identified the products of fixation as malic, citric, aspartic and glutamic acids, serine, asparagine, glutamine and tyrosine, with a very little α-keto-glutaric acid.

Kursanov, Kuzin and Mamul (6) studied the uptake of $C^{14}O_2$ by the roots of intact bean seedlings, and found that after an 18-hour exposure in the light most of the radio-activity was in the stems, indicating that the fixation products had been translocated upwards. In a later paper, Kursanov, Krjukova and Vartapjetjan (5) describe more detailed experiments in which they were able to demonstrate the fixation products of CO_2-uptake in the shoots after only 15 minutes exposure. These authors estimate that under their experimental conditions the amount of carbon dioxide absorbed by the roots is as much as 25 % of that taken up from the atmosphere by the leaves. Kursanov (4) reported later that soluble carbonates introduced into the soil together with fertilizers increased the yield of several crops by up to 18 %, and Grinfel'd (1) states that 30 or 50 Kg CO_2 per hectare, supplied as ammonium carbonate, increased the yield of sugar beets 7 and 16 % in two trials, although in the first part of the season the growth seems to have been decreased by the treatment. The Russian investigations thus appear to indicate that the uptake of CO_2 by roots is considerable and has a beneficial effect on growth.

Most of the work mentioned at the beginning on the effect of carbon dioxide and bicarbonate has been done with excised root systems, in which the translocation described by Kursanov and coworkers could not, of course, be observed. However, there is some evidence that under certain conditions an excess of bicarbonate and of carbon dioxide in the root medium may have a detrimental effect on growth, through a condition known as lime-induced chlorosis (cf experiments in (13)). Some workers have concluded that part of the deleterious effect of alkaline soils is due to the bicarbonate ion, although in general it is carbonate rather than bicarbonate which appears to exert toxic effects on roots (9). Hassan and Overstreet (2) in a study mainly seeking to relate the deleterious effects of alkali soils to the influence of sodium and other cations, did note that the growth of seedling radish roots was inhibited much more by $NaHCO_3$ than by NaCl.

As against evidence of growth-inhibition, Hoagland and Broyer, in their studies of salt uptake by excised barley roots, did not find any effect of carbon dioxide, up to 10 % in air, on the rate of salt uptake. A combination of 20 % CO_2 and 10^{-3} M HCO_3^- had no effect on the uptake of K^+ (3). Under the same conditions there was a 15 % decrease in bromide accumulation, but the authors do not consider this very significant. Contrasted with this, Steward and Preston (16), studying potato discs, found that at pH 5.5 a concentration of about 20 millimoles of bicarbonate can inhibit the bromide uptake of potato discs completely.

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[4] In particular, experiments by I. Spear and K. V. Thimann, in 1953, demonsrated that pea plants could not be grown successfully when the sole supply of CO_2 was through the root.

It is clear, therefore, that the data so far available on CO_2 uptake by the roots, and its effects on the plant, are scanty, and some of them point in opposite directions. For this reason a further study seemed well justified.

Methods

Seeds were germinated on wet filter paper and the seedlings were transplanted into gravel which had been washed several times with dilute HCl. The cultures were sub-irrigated once a day with the nutrient solution given in table I.

Table I

Composition of the Nutrient Solution

Major elements millimoles/l		Micronutrients, mg/l	
KNO_3	10	$FeSO_4 \cdot 7\ H_2O$	5
$CaSO_4$	4	$MnCl_2 \cdot H_2O$	1
$MgSO_4$	2	$Na_2B_4O_7 \cdot 10\ H_2O$	20
$Ca(H_2PO_4)_2$	1	$CuSO_4 \cdot H_2O$	0.1
$(NH_4)_2SO_4$	1	$ZnSO_4 \cdot 7\ H_2O$	0.2

The pH of the nutrient solution was 5.90. After sub-irrigation the root medium consisted of gravel : nutrient solution : air, in the proportions of 9 : 1 : 5. The control pots were flushed with air from which CO_2 had been removed, and the experimental pots with air containing various percentages of CO_2, both at the rate of 5 liters per hour per pot. It was established that the air enriched with CO_2 caused only small changes in the pH of the nutrient solution; in all cases the change was less than 0.2 pH unit. Air was obtained from a compressed air line, the CO_2 being removed from it by bubbling through 20 % KOH. Air with various percentages of CO_2 was obtained by continuous mixing with high purity CO_2. All experiments were done in a greenhouse kept at approximately 25° C.

For determination of the uptake and fixation products $C^{14}O_2$ and $HC^{14}O_3^-$ were used. Roots of intact plants were submerged in a solution containing 1 microcurie of C^{14} per ml. This solution was prepared by diluting a stock solution containing 69 mg of $Na_2C^{14}O_3$ per ml, at an activity of 0.108 millicurie per ml, with aerated tap water and then bringing the pH to 7.5 with 0.01 *N* HCl. The specific activity of the CO_2 in the final solution was 3.1 microcurie per mg CO_2; the concentration of CO_2 was 0.56 millimolar and that of HCO_3^- ions was 6.58 millimolar. The solution was in equilibrium with a partial CO_2 pressure of 1.65 %.

In treatments in which the plants were exposed to light, incandescent lamps were used and the light intensity was adjusted to about 50,000 ergs/cm^2 × sec between 400 and 700 mμ. The plants used in these experiments were between 10 and 15 days old, and had been grown in vermiculite and tap water in the greenhouse.

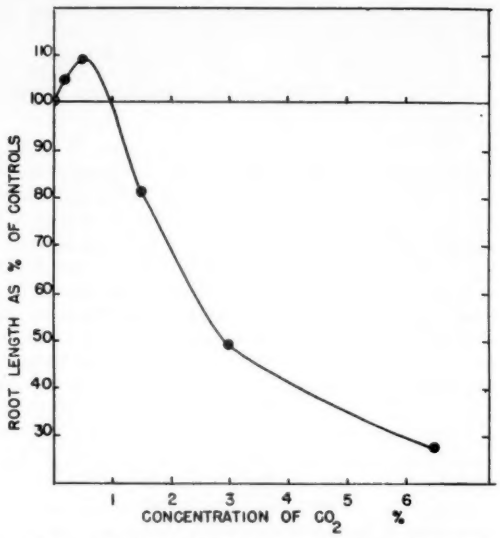

Fig. 1. Final root length of peas (*Pisum sativum* var. Alaska), after a 10- to 13-day growth period, as a function of CO_2 concentration in the root atmosphere. Each point the mean of 20 plants. Inhibition similar to that indicated at 6.5 % CO_2 was shown also by *Vicia Faba, Phaseolus vulgaris* and *Helianthus annuus*.

Results

The Effect of CO_2 and HCO_3^- on Growth of the Root System: *Pisum sativum* var. Alaska and *Avena sativa* var. Victory were used in most of these experiments. The results of a typical series of experiments, recorded after a 10- to 15-day growth period, are given in detail in table II and plotted in figures 1 and 2. Under the experimental conditions used, peas show a strong inhibition of root growth at CO_2 percentages greater than 1 %. They also show a small but significant stimulation of root growth at

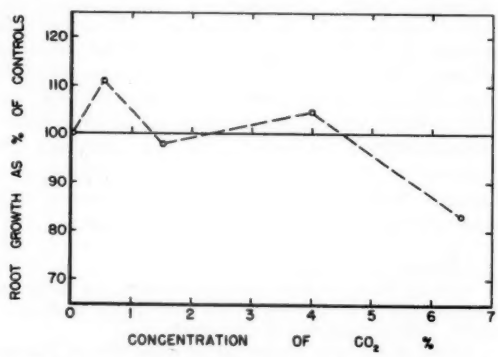

Fig. 2. Final root length of oats (*Avena sativa*, var. Segrehaver) after a 7- to 15-day period, as a function of CO_2 concentration in the root atmosphere. Similar data were obtained with barley.

TABLE II

LENGTHS OF ROOTS OF INTACT PLANTS GROWING IN LIGHT IN NUTRIENT SOLUTION SUPPLIED WITH CO_2-ENRICHED AIR

DURATION DAYS	CO_2 %	ROOT-LENGTH, CM		ACCELERATION (+) OR INHIBITION (−), %
		CONTROL IN CO_2-FREE AIR	WITH ADDED CO_2	
		Peas (Pisum sativum)		
10	0.2	19.9 ± 0.4	21.0 ± 0.3	+ 5.5
10	0.4	19.7 ± 0.6	21.2 ± 0.5	+ 7.6
11	1.5	21.8 ± 0.5	17.6 ± 0.5	−19.2
10	3.0	14.5 ± 0.3	7.5 ± 0.2	−48.3
13	6.5	21.7 ± 0.5	Died	(−73.0)
		Oats (Avena Sativa)		
12	0.6	18.4 ± 1.1	20.3 ± 2.1	+10
15	1.6	18.5 ± 1.5	18.2 ± 1.8	− 2
10	4.0	13.4 ± 0.4	14.0 ± 0.3	+ 4
12	6.5	16.7 ± 0.9	12.7 ± 1.25	−24
7	6.5	10.7 ± 0.3	9.7 ± 0.4	−10

CO_2 concentrations around 0.5 %. Oats, on the other hand, show barely significant growth responses, even at CO_2 concentrations as high as 6.5 %.

Since the curve obtained for peas shows a certain amount of distortion due to the initial length of the roots, a separate series of experiments was made in order to follow the effect of CO_2 directly on the rate of root growth. Pea seedlings, 15 days old, were planted in gravel in long glass tubes, 1.5 inches in diameter, in light, and the medium was sub-irrigated in the way described above. This method was chosen because the roots have a tendency to grow along the glass wall, making it possible to measure the growth rate of a number of roots over short time-periods. For each CO_2 concentration 4 tubes with 3 plants in each were used, and generally 3 roots measured on each plant. Thus each growth rate is based on about 36 root tips. For each CO_2 concentration a control set of plants with CO_2-free air was grown in parallel. The growth rate of these control roots varied from 0.53 to 0.62 mm per hour, except in one group where it was 0.39 mm per hour. Growth was measured over 24-hour intervals. The results, corrected for the differences in rate of the controls from one series to another, are given in figure 3. The difference between the curves of figures 1 and 3 is due to the fact that figure 1 gives only the final lengths without correction for the lengths at the start, while figure 3 presents the elongation in one 24-hour period. The small growth promotion at 0.5 and 0.7 % CO_2 is again observed. The inhibition due to higher concentrations of CO_2 is seen to be even larger than in figure 1, reaching 80 % at about 2 % CO_2.

Several other species were examined as to their response to a concentration of 6.5 % CO_2 in the root atmosphere. *Phaseolus vulgaris, Vicia Faba* and *Helianthus annuus,* grown in gravel with nutrient solution as above, all showed a complete inhibition of root growth at this concentration. *Hordeum vulgare,* six-row barley, proved to be quite unaffected, however, and thus was similar in its behavior to oats.

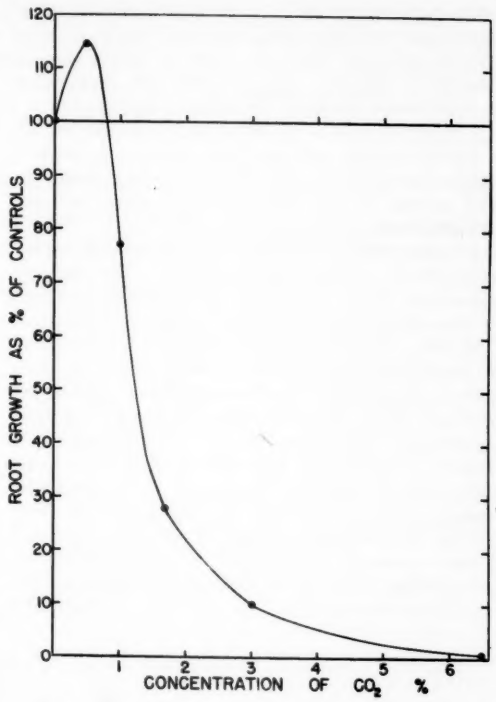

FIG. 3. Increment in root length of Alaska peas, in 24 hours, as a function of CO_2 concentration in the root atmosphere.

Assuming that the solubility coefficient for CO_2 is the same in the solution as it is in water, the Henderson-Hasselbalch equation gives the concentrations of CO_2 and HCO_3^- in the liquid root medium, at pH 5.90, shown in table III.

In the experiments described above, the inhibition of root growth occurs at much lower concentrations of CO_2 and especially of HCO_3^-, than the inhibitions reported by Porter and Thorne (13). However, these authors used a nutrient solution of much higher pH value, viz between 7.3 and 8.5, which is far from optimal for many plants, and at which the percentage of free CO_2 would be small. Similarly, in the data of

TABLE III

CALCULATED CONCENTRATIONS OF CO_2 AND BICARBONATE IN EQUILIBRIUM WITH CO_2-ENRICHED AIR IN A SOLUTION AT PH 5.90

CO_2 IN GAS STREAM, %	CO_2, MILLIMOLES/L OF SOLUTION	HCO_3^-, MILLIMOLES/L OF SOLUTION
1	0.34	0.11
2	0.68	0.22
3	1.02	0.33
4	1.36	0.44
5	1.70	0.55
6.5	2.21	0.72

Hassan and Overstreet (2), concentrations of bicarbonate ten times the highest value in table III gave only about 12% inhibition of root elongation. It seems clear, therefore, that it is the CO_2, rather than the bicarbonate, which is the effective agent.

The next step was to determine the extent of CO_2 or HCO_3^- uptake by the roots, and to investigate whether the products of fixation or their translocation could account for the difference in response between the two groups of plants.

Uptake of CO_2 and HCO_3^- by Roots; Fixation Products and Their Translocation: The root systems of five 10-day-old barley plants and five 15-day-old pea plants were submerged in 200 ml solution of $NaHC^{14}O_3$, prepared as described under Methods, and containing 1 microcurie per ml. After 24 hours exposure in the light, the roots were rinsed thoroughly with tap water, the cotyledons removed, and both root systems and shoots were killed in boiling 70% ethanol. The extracts were filtered, and the ethanol evaporated off from the filtrate, at 55° C and under vacuum. The dry residue was extracted with ether acidified with HCl, the ether extract was decanted off and evaporated, and the residues redissolved in 70% ethanol. Aliquots of the ethanol and ether extracts were then counted in a gas flow counter. The insoluble fibrous residue was dried, ground to a powder, and also counted. The results, after the usual corrections, are given in table IV, together with the dry weights of the fractions. It will be seen that the pea plants take up five to six times as much $C^{14}O_2$ as the barley. The distribution of radio-activity be-

Table IV

Distribution of C^{14} Taken Up by Roots as $C^{14}O_2$ and $HC^{14}O_3^-$, in Barley and Peas, after 24 Hours' Exposure in the Light
(All Data per 5 Plants)

Plant	Dry wt, mg	Total uptake μC	Ethanol fraction μC	Ether fraction μC	Insol fraction μC	Total uptake μC/gm dry wt
Pea shoots	117.0	0.38	0.32	0.03	0.03	3.3
Pea roots*	30.1	0.52	0.27	0.22	0.03	17.3
Barley shoots	30.0	0.08	0.04	0.03	0.01	2.7
Barley roots	20.8	0.09	0.03	0.05	0.01	4.3

* Cotyledons removed.

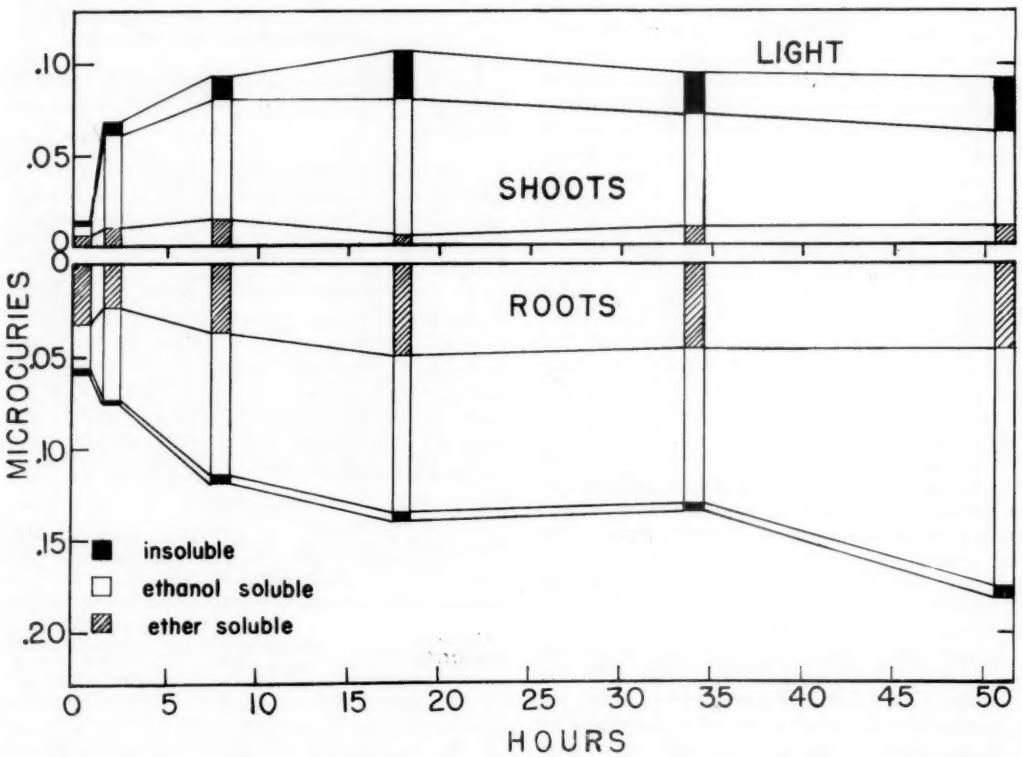

Fig. 4. Time course of uptake and distribution of C^{14} in intact pea plants in light, with the roots exposed to a solution of $C^{14}O_2$ and $HC^{14}O_3^-$.

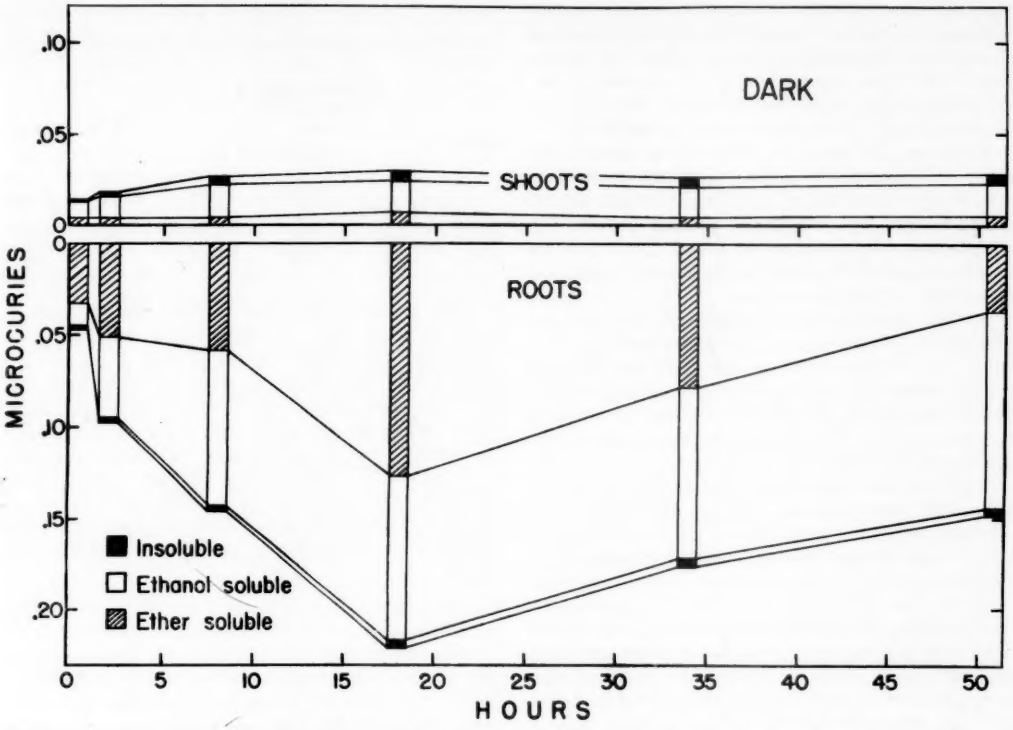

FIG. 5. Time course of uptake and distribution of C^{14} in intact pea plants in darkness, with the roots exposed to a solution of $C^{14}O_2$ and $HC^{14}O_3^-$.

tween roots and shoots is roughly equal, unlike the Russian findings (4). It is also evident that, although the ethanol fraction contains more than half the activity, the ether-soluble material is also quite active. The ether and ethanol fractions were examined by one-dimensional paper chromatography, the distribution of radioactivity on the paper being recorded with a gas flow counter and a recording count-rate meter. It was found that the ether soluble fraction of extracts of both plants had about 60 % of its total activity in malic acid, and 30 % in citric acid, although they appeared to contain a larger absolute amount of citric than of malic acid, as indicated by staining. The ethanol soluble fraction had about 55 % of its activity in sucrose, glucose and fructose, in order of decreasing importance, with the remainder distributed in several spots which were not further identified, but are probably amino acids. The alcohol insoluble fraction was not analyzed. None of the preparations obtained lost any activity as a result of treatment with HCl and subsequent heating to 70° C, indicating that very little, if any, of the absorbed carbon was present in the form of carbonate or bicarbonate.

The time course of the uptake, incorporation and translocation into the shoots, in dark and in light, was determined in peas over a period of 51 hours. The results, based on 5 plants from each determination, are summarized in figures 4 and 5. These data show that the uptake stops after about 8 hours, with 50 % of the ultimate uptake reached in about one hour. There is a considerable difference in the translocation to the shoot, depending on illumination of the shoot; in darkness the amount of translocation is very small. In the thought that the increased translocation might be due to passive uptake with the water

TABLE V

COMPARISON OF THE TRANSPIRATION OF PEA SEEDLINGS IN LIGHT AND DARKNESS WITH THEIR UPTAKE OF C^{14} FROM THE ROOT MEDIUM, AND ITS TRANSLOCATION TO THE SHOOTS

	DARK	LIGHT
Transpiration rate, mg H_2O/hr, 5 plants	215	510
C^{14} absorbed, 15-min exposure		
μC present in vol absorbed	0.05	0.13
μC uptake observed:		
Total	0.06	0.07
Shoots	0.01	0.01
C^{14} absorbed, 2-hr exposure		
μC present in vol absorbed	0.43	1.02
μC uptake observed:		
Total	0.11	0.14
Shoots	0.02	0.07

transpired, the rates of transpiration of comparable pea plants in light and in darkness were determined in a separate experiment. Table V shows the results obtained, with the implications for the uptake in the experiments described above. The data show that in 2 hours of exposure the uptake of CO_2 and HCO_3^- is much smaller than would be expected on the basis of passive absorption with the transpiration stream. Even during the period of most rapid uptake, during the first 15 minutes, the uptake of CO_2 and HCO_3^- shows no sign of selective accumulation. It is evident that the increased translocation in the light parallels the increase in transpiration rate.

In order to compare the uptake of CO_2 and bicarbonate by the roots with the respiratory production of CO_2, respiration rates of isolated root systems were determined manometrically, using plants strictly comparable to those of table V. The data of table V were calculated to CO_2-uptake per hour, and the results compared (table VI).

It will be seen that the uptake of CO_2 in peas is much larger than that in barley, if determined over a 24-hour period, as noted above. If the same comparison is made over shorter absorption periods the difference between barley and peas is smaller, indicating that the barley uptake is saturated in a shorter period of time. The general magnitude of the figures in the last column agrees well with those obtained from Poel's data (12). It should be remarked that the respiration measured is only that of the root system; the respiration of the whole plant is several times higher, and the amount of CO_2 used in photosynthesis is many times higher still, under favorable conditions. Thus it must be concluded that, under the experimental conditions used here, the uptake of CO_2 by the roots must be considerably less than 1 % of the amount of CO_2 taken up by the leaves in photosynthesis.

After the present study was completed, Miller and Evans (10) reported that bicarbonate ions inhibit the activity of cytochrome c oxidase. This finding raised the possibility that the difference between barley and peas in their response to CO_2 and HCO_3^- might be due to a difference in the terminal oxidases of the roots. Since the most characteristic property of cytochrome oxidase is the light-reversible inhibition by CO, a few measurements of the effect of CO in dark and in light on the respiration of excised root systems of both plants were made. Whole root systems in moist air were used. In both barley and peas, root respiration was inhibited about 60 % in a gas mixture of 90 % CO and 10 % O_2, while in white light this inhibition was reduced to 20 % (table VII). These results, while not extensive enough for detailed analysis, indicate that in both plants cytochrome c oxidase is at least the predominant oxidase. Apparently, therefore, the inhibition of cytochrome oxidase reported by Miller and Evans does not account for the difference in sensitivity to bicarbonate of these two plants.

TABLE VI

UPTAKE OF C^{14} BY ROOTS COMPARED WITH RESPIRATION RATE OF THE ROOT SYSTEM

PLANT	RESPIRATION	UPTAKE BY THE ROOTS $\mu L\ CO_2$/HR	$\frac{\text{UPTAKE}}{\text{RESP.}} \times 100$
Pea	96	6.0	6.2
Barley	53	1.1	2.1

Both data calculated to microliters CO_2 per 5 plants per hour. Period 24 hours. Solution: $C^{14}O_2 + HC^{14}O_3^-$ as in text.

TABLE VII

LIGHT-REVERSIBLE INHIBITION OF ROOT RESPIRATION BY CO

ROOTS	O_2 CONSUMPTION, $\mu L\ O_2$/HR × GM FRESH WT			% INHIBITION	
	AIR	CO/O_2 (9:1)			
		LIGHT	DARK	LIGHT	DARK
Barley	369	300	161	19	56
Pea	303	243	142	20	53

DISCUSSION

The experiments described above clearly indicate that relatively low concentrations of CO_2 and HCO_3^- in the root medium strongly inhibit root growth in peas, while less extensive data show the same thing for three other dicotyledenous plants. The same levels of CO_2 and HCO_3^- have no appreciable effect on the two cereals. It might be noted also that the uptake of ions by barley roots (3) appears more resistant than that by potato (16), though the two researches involved are not very comparable. The amounts of carbon dioxide and bicarbonate taken up by representatives of the two types of plants are different (table VI), but the difference hardly seems great enough to account for the complete absence of inhibition in the cereals. Furthermore, there does not seem to be any major difference in the types of fixation products, nor in their translocation into the shoots. A differential effect on the terminal oxidase appears to be ruled out. The small amount of CO_2 taken up must be incorporated into other compounds almost immediately, since none of it is found as carbonate. The effect is not due to formation of bicarbonate, as was suggested by Lindsay and Thorne (7) for the effect of CO_2 in increasing chlorosis, because the concentrations of bicarbonate ion used in the present experiments were less than 1 millimolar. It is thus apparently due to CO_2 itself. It seems, therefore, that some specific toxic effect, to which oats and barley are immune, is exerted by CO_2 in the dicotyledons.

The actual amounts taken up through the roots are in good agreement with estimates derived from the findings of Overstreet et al (11), and of Poel (12). It is not inconceivable that the CO_2 produced in res-

piration arises closer to the fixing sites in the roots than the CO_2 being absorbed from the root medium, with the result that the technique used might yield too low an estimate of the fixation of CO_2. However, the resulting error in the interpretation of the data obtained with $C^{14}O_2$ cannot be very large, since such preferential recirculation of respiratory CO_2 would result in deviations from unity in the respiratory quotient, which are not observed. The absence of appreciable amounts of carbonate or bicarbonate contrasts with the experience of Smith and Cowie (15) with sunflower leaves, in which much of the CO_2 fixed was in reversible combination, which was ascribed to bicarbonate.

The fate of the fixation products, as far as they were determined, seems to be compatible with the pathways suggested by Kursanov (4), but the importance of CO_2 taken up from the root medium appears to be considerably smaller than Kursanov has concluded. Furthermore, Kursanov does not mention the inhibiting effect of relatively low concentrations of CO_2 and HCO_3^- in many species. It should be noted that in both types of plants the uptake ceases after a relatively short time, and the major fixation products are compounds already present in much higher concentrations under normal conditions, so that only a very small increase in their concentration is likely to occur.

In their experiments which led to increased yields of beans, barley and sugarbeet, Kursanov and coworkers (4, 5 and 6) added soluble carbonates in the form of fertilizer, presumably in amounts not exceeding a few hundred pounds per acre. It is interesting to compare this with the normal production of CO_2 by typical soils. Determinations made by Lundegårdh (8) showed that the CO_2 production, in mg per hour per square meter, ranged from 452 in garden soil to 671 in a sandy loam, in a study covering a large number of soil types. Taking an average figure of 600 mg per hour per square meter, this would correspond to about 4000 lbs of CO_2 per acre per month, or well over 10,000 lbs in a growing season. It seems unlikely, therefore, that the increases in yield mentioned by Kursanov could be due specifically to the carbonate added. It is not clear either that the influence of the nitrogen added as ammonium was completely ruled out, especially in Grinfel'd's experiments (1). These facts, taken together, make it improbable, unfortunately, that fertilization with bicarbonate could have much agricultural value.

The concentration of bicarbonate used in the C^{14}-uptake experiments, 6 millimolar, is comparable to the concentrations recorded in soil solutions from agricultural soils, while soil gases may contain from 0.15 to over 2.5 % CO_2 and figures as high as 12 % have been recorded (8, 18). It follows, therefore, that the levels of CO_2 and bicarbonate in the soil must often be high enough to inhibit root growth of dicotyledons, and it may well be that much of the benefit of frequent cultivation of crops derives from the improved diffusion of CO_2 from the soil into the atmosphere. Cereals, on the other hand, judging from their lack of response to high CO_2 concentrations, would appear not to need cultivation for this reason.

Summary

The growth of roots of *Pisum sativum*, *Vicia Faba*, *Phaseolus vulgaris* and *Helianthus annuus* is completely inhibited if the root medium is aerated with 6.5 % CO_2 in air. *Avena sativa* and *Hordeum vulgare* are unaffected by such a treatment. Peas show a small but consistent stimulation of root growth when the root atmosphere contains 0.5 % CO_2, but are clearly inhibited at levels as low as 1.5 %. Some possible explanations of the difference in sensitivity to CO_2 of peas and barley have been ruled out, and a specific toxic effect seems indicated.

The uptake of CO_2 by the roots of both peas and barley is of the order of only a few percent of the amount produced by respiration in short term experiments, and it virtually ceases after about 8 hours. The bulk of CO_2 fixed was converted to malic and citric acids and sugars; after 24 hours only about 10 % had entered the alcohol-insoluble fraction.

Translocation of the products into the shoots of the intact plant was about 3 times as great in the light as in the dark.

It is concluded that carbonate fertilization of crops is unlikely to be beneficial, and that the CO_2 content of some soils may, indeed, already be supra-optimal.

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SULFHYDRYLS IN PLANTS. I. REACTIONS WITH GROWTH REGULATORS[1]

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Many studies have indicated a close connection between sulfhydryl substances and plant growth processes (21). For example, many growth inhibitors are able to combine with sulfhydryls. Several recent studies have led to suggestions of various schemes which involve sulfhydryls in the possible mechanisms of auxin action (8, 12, 17, 21, 23). While there is much interest in sulfhydryls, very little is known concerning their occurrence in plants and their changes during growth (2, 20).

The present work was undertaken to study plant sulfhydryl substances as they may be related to growth. This first paper is an examination of some non-enzymatic reactions with growth regulators. A preliminary report on part of this material has appeared elsewhere (9).

Materials and Methods

Sources of the various materials included: maleimides kindly donated by Dr. J. van Overbeek; chelidonic acid originally prepared by Dr. E. Ramstad and recrystalized from water; CoA[3] donated by the Pabst Laboratories; phosphotransacetylase donated by Dr. E. R. Stadtman through the courtesy of Dr. H. Beevers. All other reagents were obtained commercially. The TIBA was treated with charcoal and recrystallized from ethanol.

Sulfhydryl compounds were made up fresh daily and kept chilled until use. For reactions in ordinary test tubes, the sulfhydryl was added last, the tubes placed in a desiccator and evacuated. Thunberg tubes were used as a further precaution against error due to autoxidation. In the latter case the sulfhydryl was maintained in dilute acid and not exposed to other reagents until the oxygen had been expelled. With both methods the systems were alternately evacuated and filled with nitrogen three times.

Sulfhydryl estimations were obtained by the nitroprusside test (6). A typical analysis consisted of mixing 0.5 ml of sample with 5 ml saturated NaCl and 1.0 ml 2 % sodium nitroprusside, followed by 1.0 ml of a mixture of 1.5 M sodium carbonate and 0.025 M sodium cyanide. The extinction of the resulting violet color was then determined at 520 mμ on a Bausch and Lomb "Spectronic 20" or Beckman DU spectrophotometer. With the Thunberg tube technique it was possible to complete the sulfhydryl analysis within less than 1 minute after the tube was opened; with the desiccator method a batch of tubes were exposed to air while analyses were proceeding. Variations due presumably to autoxidations were consequently greater with the latter method.

CoA was measured by the phosphotransacetylase assay of Stadtman (19).

Chromatographic techniques and solvent systems used were those of Gutcho and Laufer (7). The ascending method was used. Since temperature control was not obtained, there was some variation in R_f values from one run to another.

Experimental

A survey of various growth regulators led to the finding that TIBA reacts non-enzymatically with such

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[3] Abbreviations employed are as follows: 2,4-D, 2,4,-dichlorophenoxyacetic acid; BAL, 2,3-dithiopropanol; CoA, coenzyme A; GSH, glutathione; GSSG, oxidized glutathione; TIBA, 2,3,5-triiodobenzoic acid; TRIS, tris (hydroxymethyl) aminomethane.

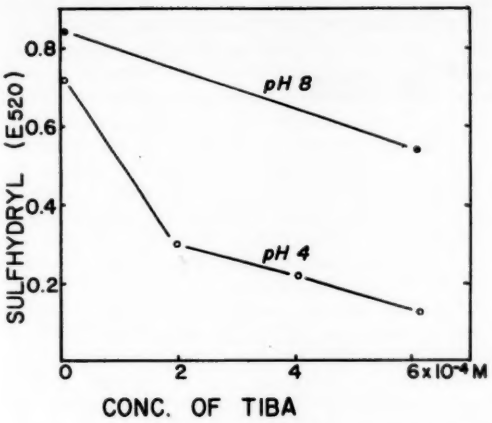

FIG. 1. The effect of TIBA on free sulfhydryls of GSH. Thunberg tube system: 2 ml TIBA, into which 4 micromoles GSH in 0.4 ml McIlwaine buffer pH 4 or M TRIS pH 8 is tipped after evacuation. Nitroprusside test after 24 hours.

sulfhydryl substances as cysteine, glutathione, and coenzyme A. Incubation of glutathione with TIBA at room temperature in vacuo for five hours results in marked decreases in free sulfhydryl content as indicated by the nitroprusside test. A sample experiment is presented in figure 1. It can be seen that the reaction proceeds more readily at pH 4 than at pH 8. The reaction was not linear with time, nor was all of the -SH removed after 24 hours with nearly two-fold excess of TIBA. The time course of the reaction was determined in an experiment described in table I. The disappearance of -SH was found to be complete with four-fold excess of TIBA after 24 hours. The reaction proceeds at a moderately slow rate, a 50 % decrease in free -SH of equimolar GSH and TIBA being obtained after about 8 hours at pH 4. As a comparison, the reaction of GSH with iodoacetate is favored by alkaline pH and the reaction proceeds at least ten times faster (18).

The observed results could have been due to either a coupled oxidoreduction or a condensation. The latter alternative was proved by chromatographic analysis as shown in figure 2. Samples from the Thunberg tubes of the time course experiment (table I) were chromatographed in phenol-water. Since glutathione contains an α-amino acid group, any compound formed with TIBA could be detected by the appearance of an additional spot with ninhydrin. After one hour of incubation a new spot appeared above that of GSH. The spot increased in intensity with time while glutathione disappeared, and at 24 hours the glutathione had been converted entirely to the new compound (fig 2). Superior separation of the reaction product was obtained with several other solvent systems as shown in table II.

Clearly glutathione was converted by TIBA to another amino acid–containing compound, but the possibility had not been excluded that a contaminant in the sample of TIBA might have been responsible.

TABLE I

TIME COURSE OF THE REACTION BETWEEN TIBA AND GLUTATHIONE

TIME (HRS)	NITROPRUSSIDE REACTION (E 520 mμ)	
	WATER	TIBA
0	0.660	....
1	....	0.580
4	....	0.420
8	0.680	0.323
24	0.680	0.000

Thunberg tube system: 2 ml water or 10^{-2} M TIBA neutralized with NH_4OH; into which is tipped 0.4 ml 10^{-2} M of GSH in 0.3 M McIlwaine's buffer pH 4.

TABLE II

R_f VALUES FOR GLUTATHIONE, OXIDIZED GLUTATHIONE, AND THE GSH-TIBA REACTION PRODUCT IN SEVERAL SOLVENT SYSTEMS

COMPONENT	R_f VALUES			
	PhW	IFW	ETBFW	TBFW
GSH	0.36	0.52	0.51	0.35
GSSG	0.05	0.30	0.25	0.05
GSH + TIBA	0.37	0.84	0.71	0.58

PhW: phenol water
IFW: isopropanol–formic acid–water, 65 : 1 : 34
ETBFW: ethanol–t butanol–formic acid–water, 60 : 20 : 5 : 15
TBFW: t butanol–formic acid–water, 70 : 15 : 15

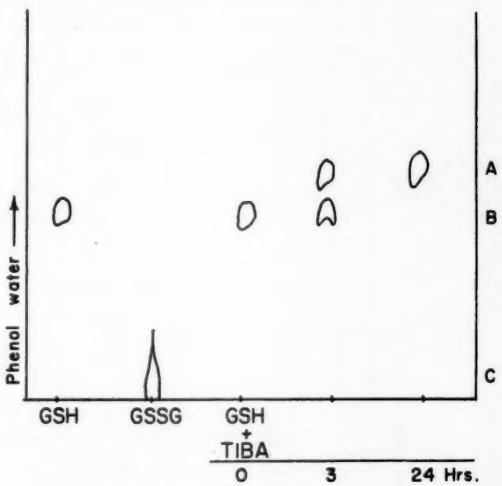

FIG. 2. The formation of a new compound upon reaction of TIBA with glutathione. Experiment as in table I. Chromatographed in phenol-water. Solvent flowed beyond end of papers; for normal R_f values, see table II.

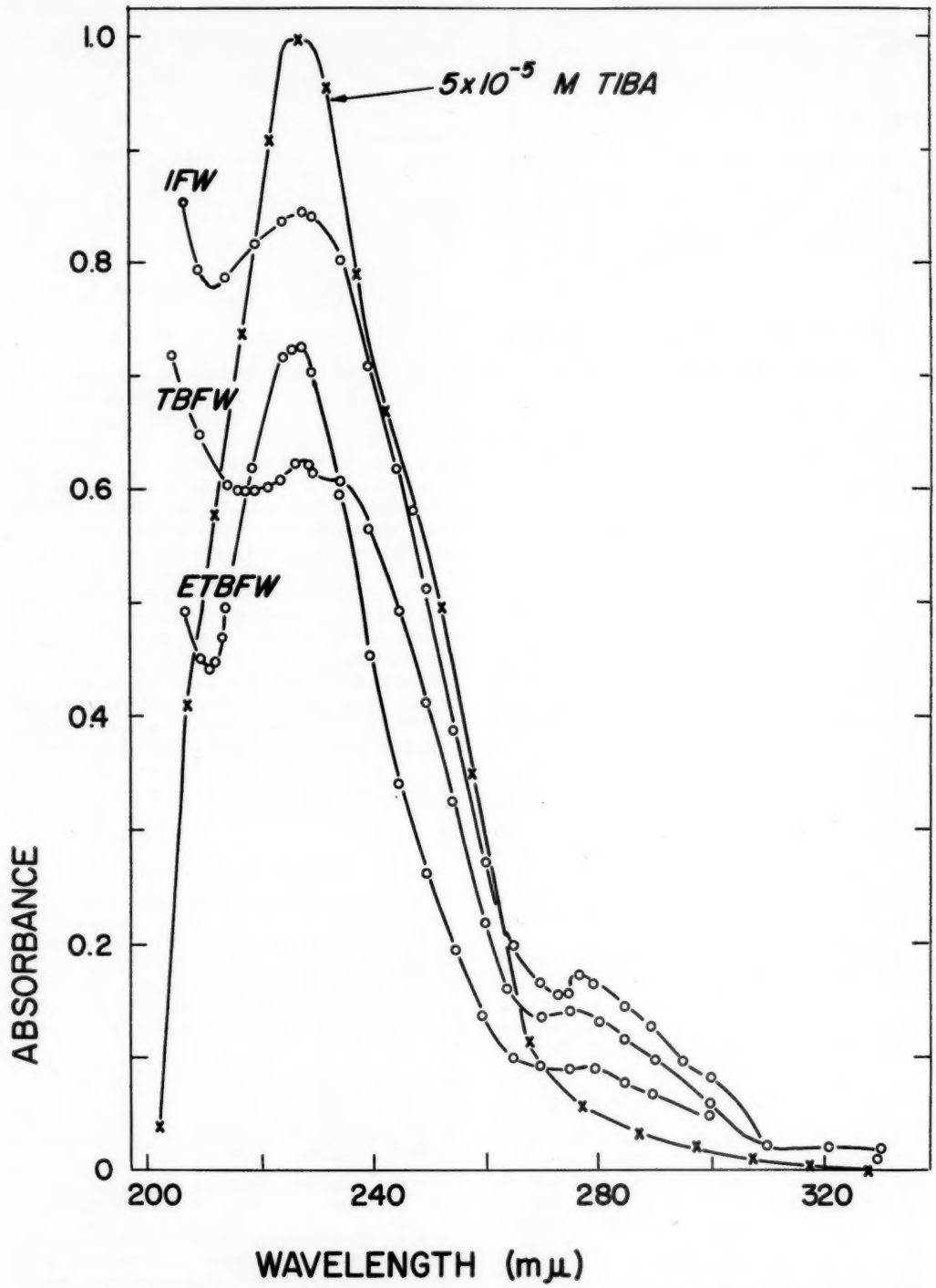

FIG. 3. Ultra-violet absorption spectra of TIBA and its reaction product with glutathione. Samples eluted from filter paper after development in various solvents (abbreviations as in table II).

That a true condensation between TIBA and GSH had occurred was indicated by the finding that the new material contained an aromatic nucleus. Large (100 μl) quantities of the TIBA-GSH reaction mixture were chromatographed in several solvent systems, the new component cut out, eluted, and the absorption spectrum determined. As shown in figure 3, the new compound separated in any of three solvent systems has a clear maximum at about 228 mμ which is very similar to that of TIBA itself. There is in addition a small peak or shoulder at about 277 mμ which does not correspond to either of the reactants.

The non-enzymatic reaction between TIBA and sulfhydryl compounds appears to occur generally; a reaction with protein sulfhydryl has been obtained (16) and also a reaction with the -SH group of CoA. A more detailed account of the latter reaction has been described elsewhere (9) and only a brief example will be presented here. A 100-fold excess of TIBA was incubated with coenzyme A for 60 minutes and the activity of the CoA was then assayed by the phosphotransacetylase system (19) as shown in table III. There was a sharp loss in the catalytic activity of the coenzyme. Although the reaction was allowed to proceed in air, only a small recovery was obtained with the addition of cysteine at the end of the reaction period. In the same experiment an aliquot of CoA incubated with 2,4-D showed a more marked recovery with the addition of cysteine, indicating that in this case the lowered CoA activity was due to an oxidation, probably catalyzed by metal impurities.

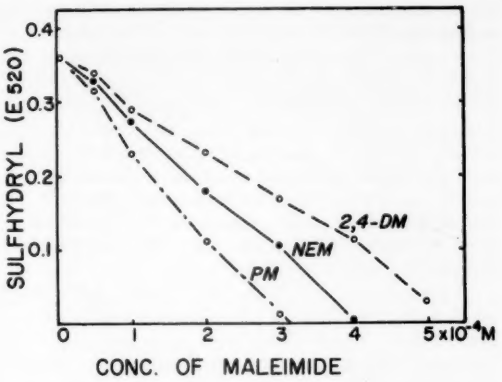

FIG. 4. Reaction of N-substituted maleimides with the sulfhydryl of glutathione. System: 4×10^{-4} M GSH, 1.5 M McIlwaine buffer pH 8, and maleimide as indicated. Final volume 1.0 ml. Held 30 min at 20° C before nitroprusside test.

Maleimides are known to react with sulfhydryl groups (4, 5) and N-aryl derivatives have been used as growth regulators (15). Their reactions with sulfhydryls in the present system was examined. N-ethyl maleimide, N-(2,4-dichlorophenyl) maleimide, and N-phenyl maleimide were added to GSH under nitrogen and the disappearance of free sulfhydryls was followed with the nitroprusside test as shown in figure 4. The various derivatives seem to be similarly effective in causing SH disappearance. When equimolar amounts were added to GSH essentially complete disappearance of the SH resulted after only a few minutes at a neutral or alkaline pH.

The addition products of maleimides with sulfhydryl substances can be detected by paper chromatography. For example, in phenol-water the product with 2,4-D maleimide runs ahead of GSH, having an

TABLE III

EFFECT OF TIBA AND 2,4-D ON ACTIVITY OF COENZYME A IN THE PHOSPHOTRANSACETYLASE SYSTEM

ADDITIONS	FINAL CONC OF CoA AFTER INCUBATION			
	NO CYSTEINE		+ 0.01 M CYSTEINE	
	Micro-mole/ml	*% Control*	*Micro-mole/ml*	*% Control*
None	0.042	(100)	0.048	(100)
5×10^{-5} M TIBA	0.036	86	....	...
5×10^{-4} M TIBA	0.027	64	0.039	81
5×10^{-3} M TIBA	0.001	3	0.010	21
5×10^{-4} M 2,4-D	0.035	82	....	...
5×10^{-3} M 2,4-D	0.032	76	0.046	97

4.8×10^{-5} M CoA incubated for 60 minutes with TIBA or 2,4-D in 0.05 M TRIS buffer, pH 8; CoA assayed after incubation. Cysteine added at end of incubation where indicated.

TABLE IV

INFLUENCE OF VARIOUS SUBSTANCES UPON THE SULFHYDRYL OF GLUTATHIONE

GROWTH REGULATOR	CONCENTRATION	NITROPRUSSIDE REACTION (E 520 mμ)				
		NO BUFFER	pH 4	pH 5	pH 6	pH 7
GSH alone	.....	0.53	0.47	0.47	0.45	0.43
Cupric sulfate	5×10^{-5} M	0.47	0.35	0.27	0.35	0.33
Iodoacetic acid	5×10^{-3} M	0.40	0.42	0.38	0.32	0.04
Naphthaleneacetic acid	5×10^{-4} M	0.48	0.47	0.45	0.43	0.41
Maleic hydrazide	5×10^{-3} M	0.50	0.45	0.44	0.45	0.44
p-Chlorophenoxy acetic acid	5×10^{-4} M	0.33	0.48	0.45	...	0.40
Chelidonic acid	5×10^{-3} M	0.62	0.60	0.47	0.52	0.46

Glutathione 5×10^{-4} M throughout; 1.5 M McIlwaine's buffer and reagents in 1.0 ml; incubated under nitrogen 5 hrs at room temperature.

R_f of 0.84 to 0.94, and the product with N-ethyl maleimide, 0.70.

A diverse array of other growth substances was tested, but none were found to have indisputable reactivity toward sulfhydryls. Sample data using the nitroprusside test to detect free sulfhydryls are presented in table IV. In this experiment the incubation was performed under nitrogen in a desiccator, but strictly anaerobic conditions were not obtained. It can be seen that while iodoacetate was highly effective, chelidonic acid, maleic hydrazide, and two auxins were without a significant effect.

Several exploratory experiments indicated a slight reaction between certain other growth regulators and glutathione. These effects were examined under the rigorous conditions of the Thunberg tube. It was found that 2,4-D and 2,4-dichloranisole reacted regularly to a small extent with the -SH of glutathione, but the effects were slight and may have been due to contaminants.

It was a matter of some interest to discover if conditions would be found for a reaction between coumarin and sulfhydryls. This and related lactones are growth inhibitors and preventions of their inhibitions with added sulfhydryls have been reported (10, 22). We have reported preliminary experiments suggesting that a reaction occurred (9), but these findings were not confirmed under the rigorous conditions of the Thunberg tube method. The role of coumarin as a growth inhibitor thus remains obscure.

Discussion

The suggestion that auxin combines in the cell to the cysteinyl -SH of some protein (12) has been widely quoted, but direct evidence for such a reaction has not been produced. In the experiments reported here, a non-enzymatic condensation between sulfhydryls and the auxin synergist, TIBA, has been established. This does not seem to be a reaction common to the auxins, for none of the growth regulators other than those identified as "sulfhydryl inhibitors" react with simple sulfhydryls such as glutathione at physiological temperatures and pH.

If the TIBA -SH reaction is compared with the iodoacetate -SH reaction, it is seen that the rate of TIBA reaction is markedly slower and it is promoted by acidic rather than alkaline conditions (18). The TIBA reaction involves a condensation of the aromatic nucleus of TIBA with the sulfur atom, presumably forming a thio-ether.

The inactivation of sulfhydryl groups by TIBA in vivo will be reported elsewhere (16). It is pertinent to point out that a strong inhibition of auxin transport is obtained (14) as well as with classical -SH combining reagents (13). These facts suggest that alteration of auxin systems in plants by TIBA may be due in part to its property of reacting with sulfhydryls.

The actions of several growth regulators have been interpreted as being due to sulfhydryl inactivation. With both coumarin (22) and chelidonic acid (10), the evidence adduced was the prevention of growth inhibition by the simultaneous addition of a -SH substance such as BAL, GSH or cysteine. In the present experiments no indication of in vitro reactions of these substances with glutathione or cysteine was found. In an early chemical study Cavallito and Haskell (3) also failed to obtain any reaction of coumarin with cysteine. A related compound, protoanemonin, which has stronger action as a growth inhibitor (22), differs from coumarin in having an ethylenic double bond conjugated with the oxo group of the lactone; condensation with a sulfhydryl in this case would seem more probable.

The lack of reaction of the auxins tested with sulfhydryls does not exclude the possibility of such a combination in vivo. Indeed, the synthesis of indoleaspartate from indoleacetic acid in the pea stem (1) may very likely occur through an indoleacetyl-S-CoA, as Andreae and van Ysselstein suggest. Millerd and Bonner (11) reported a slight formation of such auxin-CoA derivatives by an enzyme preparation from mung bean mitochondria, but we found that the small changes observed in a similar system could be accounted for as oxidations (9).

Summary

Non-enzymatic reactions of the sulfhydryl group of glutathione were studied using simple in vitro conditions. The following conclusions were drawn:

1. TIBA (2,3,5-triiodobenzoic acid) reacts with the sulfhydryl, as shown by disappearance of the nitroprusside color and the appearance of a new addition product containing both the ninhydrin positive groups of the glutathione and the aromatic ring of the TIBA. The reaction with TIBA was shown to inactivate coenzyme A.

2. Other sulfhydryl inactivators or suspected inactivators were examined for this property, and it was found that sulfhydryl disappearance occurs with maleimides, iodoacetate and *p*-chloromercuric benzoic acid. No reaction under the conditions of these tests was obtained with coumarin, chelidonic acid, maleic hydrazide, or a variety of auxins.

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SULFHYDRYLS IN PLANTS. II. DISTRIBUTION IN TISSUES[1,2]

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The first study in this series (12) was concerned with chemical as distinct from biological reactions of plant growth regulators with -SH groups. Before information obtained could be applied to tissue systems, it was necessary that means be available for the estimation of the sulfhydryl contents of plants. While assays for specific sulfhydryl compounds in plants have been performed (24), few estimates of total sulfhydryl content have been forthcoming. Polarographic methods have shown great promise both for nonprotein (26) and protein (3) sulfhydryls. Silver titration of sulfhydryls in homogenates with the platinum electrode has been reported for lily anthers (23), barley seeds (5), and bacterial extracts (14, 16). The results of a colorimetric assay (6) of sulfhydryls in bean leaves has also been briefly reported (9).

The present study is principally concerned with the estimation by amperometric titration of total sulfhydryl including the protein fractions of etiolated pea seedlings and mature bean leaves. A preliminary report has appeared elsewhere (20).

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METHODS AND MATERIALS

The procedure used for estimating -SH contents was based on the argentammine method of Kolthoff and Harris (10) in alcoholic ammonia and, as modified by Benesch et al (3), in aqueous TRIS (tris(hydroxymethyl)-aminomethane) buffer.

In the present study the solutions to be analyzed were stirred magnetically in 50-ml beakers (cf. 22). The bottoms were blown out slightly to form a depression in which a 2-cm teflon-covered stirring bar rotated smoothly. The procedure was otherwise similar to that of Benesch et al (3). A Rubicon (Philadelphia, Pa.) galvanometer was employed with a sensitivity of 0.001 μa per mm, and the galvanometer circuit was critically damped. Silver nitrate (0.01 M)

was added in 1-μl increments from an "Agla" micrometer syringe (Burroughs Wellcome and Co., Inc., Tuckahoe, New York) fitted with a 10-cm bent glass delivery needle.

The test material included etiolated *Pisum sativum* and *Avena sativa* seedlings as used for auxin assays, *Helianthus annuus* internodes obtained from plants grown in the greenhouse, as in auxin transport experiments (15), and the mature leaves of *Phaseolus vulgaris* grown in the greenhouse.

Tissue Sections: Cut sections of the tissue were washed on a coarse sintered glass funnel and ground in a conical tissue grinder with 5 ml of a mixture of 0.15 M TRIS nitrate, pH 7.4, 0.01 M KCl, with or without the addition of 8 M urea. The latter served as a mild denaturing agent, providing an estimate of the total -SH available to the tissue. The homogenate was then drained through a 30- or 60-ml Buchner-type coarse sintered glass funnel with an additional 25 ml of the TRIS mix. This step was essential to free the sample from cell wall debris which otherwise fouled the platinum electrode. A separate funnel was required for each sample, since otherwise filtration was incomplete. The filtered homogenate was finally transferred to a specially prepared 50-ml beaker (described above) and the titration begun. The whole operation from washing the sections to the beginning of the titration required three to five minutes. A standard interval between grinding and the addition of silver nitrate was chosen for each experiment.

Subcellular Fractions: The fractionation methods corresponded to those commonly employed for the preparation of mitochondria (8). Chilled tissue was blended for 15 seconds in a chilled Waring blendor with 2 × to 5 × (v/w) of a frozen slush of 0.3 M sucrose and 0.05 M potassium phosphate, pH 7. With bean leaves, 0.01 M $MgCl_2$ was included. The resulting suspension was strained through cheesecloth, thus separating the gross cell wall residue from the "homogenate." The residue was then re-extracted with a small volume of the medium. The homogenate was subjected to fractional centrifugation, as indicated in the text, in a Servall SS-1 centrifuge in a 0 to 2° C cold room. Non-protein components of the various pea and bean fractions were obtained by adding 30 % sulfosalicylic acid to 3 % final concentration, centrifuging, and collecting the supernatants. The -SH contents of all samples were then determined by titration in a mixture of 95 % ethanol, 0.25 M ammonium hydroxide, 0.05 M ammonium nitrate, and 10^{-5} M ethylenediaminetetraacetic acid (10). Nitrogen analyses on the various samples were obtained by micro-Nesslerization (11).

Results

Application of the Method to Plant Material: Of the methods available for estimation of sulfhydryl groups (17), amperometric titration proved the most suitable. Because of interfering substances found in unpurified plant extracts, the nitroprusside test (7) had to be excluded. The optical method of Boyer (4) employing *p*-chloromercuribenzoate was not practicable because of the high extinction of the plant extract. Amperometric titration with amino (3) or ammoniacal (10) silver ion, on the other hand, is not influenced by the color or turbidity of the extract, is highly specific (1, 3), sensitive, and yielded reproducible values. Recovery of cysteine or glutathione added to homogenates in alcohol-ammonia was quantitative.

With pea stem sections in the TRIS mix, a partial destruction of sulfhydryl groups occurred between grinding and the addition of silver ion. An experiment in which pea stem section homogenates were allowed to incubate from 4 to 30 minutes at room temperature indicated a logarithmic decline in the sulfhydryl content. Extrapolation to zero time indicated at four minutes a loss of 12 % of the initial -SH; as it was technically not possible to decrease the interval, no information was available on the actual losses in the moments following disruption of the tissue. When pea sections were ground in TRIS with added glutathione the -SH recovered at four minutes was also 12 % less than theoretical. Since the use of a standard interval between grinding and titration yielded closely reproducible values, this destruction of sulfhydryls proved a relatively minor problem. The use of TRIS retains a distinct advantage over alcoholic ammonia; for the degree of denaturation of protein can be more closely controlled (3).

Since it was planned to use the sulfhydryl assay

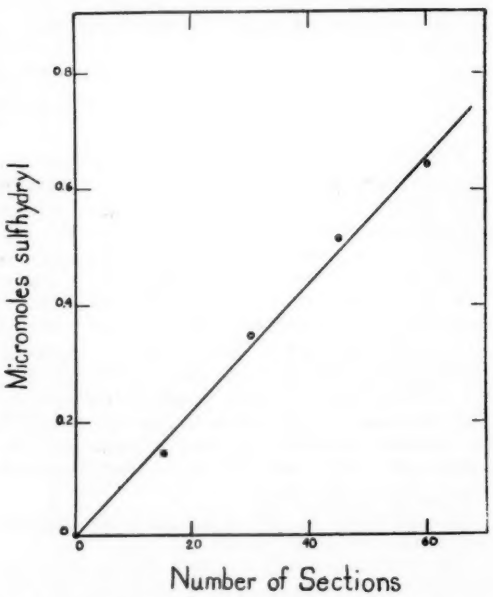

Fig. 1. Sensitivity of titrations with pea stem homogenates. -SH content of pea stem section homogenates determined for various sizes of sample. Sections homogenized and titrated in 0.15 M TRIS nitrate, pH 7.4, 0.01 M KCL, and 8 M urea.

in conjunction with growth studies, it was essential that the method be sufficiently sensitive for small quantities of tissue. Sample titrations were, therefore, performed with varying quantities of etiolated pea stem sections. The results are illustrated in figure 1. It can be seen that the assay is linear to at least sixty 10-mm sections. Thirty sections or about 600 mg fresh weight provided convenient sulfhydryl values.

SULFHYDRYL CONTENT OF TISSUES: Oat coleoptile sections and pea stem sections were ground and titrated in TRIS-nitrate mixture containing 8 M urea. The results are summarized in table I. Included is a measurement of 6-cm sunflower internodes, kindly prepared by Dr. Ethel Niedergang-Kamien (15).

TABLE I

SULFHYDRYL CONTENT OF AVENA, HELIANTHUS AND PISUM TISSUES

PLANT	AGE	TISSUE	-SH CONTENT	
			MICROMOLES/GM FRESH WT	MICROMOLES/100 SECTIONS (10 MM)
Avena sativa (oat)	72 hrs	Etiolated coleoptiles	0.32	0.46
Helianthus annuus (sunflower)	3 wks	Internodes	0.08	0.16
Pisum sativum (pea)	7 days	Etiolated subapical internodes	0.86	1.41 ± 0.08 *

-SH of whole tissue extract estimated by amperometric titration in TRIS-8 M urea medium. See Methods.
* Twenty-seven separate experiments.

The disparity in -SH content among the three tissues was striking. Pea stem tissue was 2.5 times richer on a fresh weight basis than the coleoptiles and nearly 10-fold richer than sunflower stems. This brings immediately to mind the high sensitivity toward sulfhydryl-combining reagents of Avena relative to Pisum tissue (25). On a per section basis the disparity is even greater, since pea stems are heavier than the hollow coleoptiles.

SULFHYDRYL CONTENT OF SUBCELLULAR FRACTIONS: The tissue fractions were prepared and analyzed for sulfhydryl content as described under Methods. Since the interval between grinding and titration could not be closely controlled, the titrations were performed in alcoholic ammonia.

In the experiment summarized in table II, the pea stem homogenate was separated into one "soluble" and four particulate fractions, sedimenting at 500, 5000, 50,000, and 500,000 g-minutes with one washing each. These corresponded to starch, plastids, and two fractions of mitochondria as judged by visual inspection. The majority of the total and nearly all of the non-protein -SH was recovered there. The protein -SH appeared to be divided approximately in proportion to the quantity of protein in the fraction.

In another experiment the mature leaves of the red kidney bean were examined. Leaves have been reported to be resistant to clean fractionation (13), and the mitochondrial fractions in these experiments were indeed contaminated with broken chloroplasts.

Fresh bean leaves were collected from the greenhouse, 20 grams weighed out and blended as described above. When a sample of the homogenate was centrifuged for 500,000 g-minutes, layering into three bands was observed in the sediment. It was found that these bands could be separated by varying the force-time integrals. A fraction, mostly starch, was col-

TABLE II

INTRACELLULAR FRACTIONATION OF -SH CONTENT OF PISUM SEEDLINGS

FRACTION	APPROX. FORCE-TIME INTEGRAL DURING CENTRIFUGATION (G-MIN)	NITROGEN CONTENT				-SH CONTENT				% OF HOMOGENATE	
		MG N/GM FRESH WT		% OF HOMOGENATE		MICROMOLES/GM FRESH WT		MICROMOLES/MG N × 10^2			
		TOTAL	NON-PROTEIN	TOTAL	NON-PROTEIN	TOTAL	NON-PROTEIN	TOTAL	NON-PROTEIN	TOTAL	NON-PROTEIN
Homogenate	.....	12.3	2.56	100	100	1.96	0.33	16	13	100	100
Starch	500 *	0.29	0.07	2.4	3	0.03	0.03	12	39	1.8	9
"Plastid"	4,500 **	1.88	0.60	15.3	23	0.11	0.01	6	17	5.6	3
Mitochondria A	50,000 †	0.93	0.18	7.6	7	0.09	0.01	9	8	4.3	3
Mitochondria B	500,000 ††	0.71	0.08	5.8	3	0.11	0.02	15	23	5.6	6
"Soluble"	500,000 ††	7.44	1.74	60.5	68	1.20	0.28	16	16	61.2	85
Recovery	.....	....	...	91.6	104	...	...	..	..	78.5	106

Fractions prepared from sucrose-phosphate homogenates of 3-day-old etiolated seedlings. -SH determined by titration in alcohol ammonia.
* 1000 × g for 30 sec.
** 1500 × g for 5 min.
† 10,000 × g for 5 min.
†† 16,700 × g for 30 min.

TABLE III

INTRACELLULAR FRACTIONATION OF -SH CONTENT OF PHASEOLUS VULGARIS LEAVES

FRACTION	APPROX. FORCE-TIME INTEGRAL IN CENTRIFUGATION (G-MIN)	NITROGEN CONTENT		-SH CONTENT			
				MICROMOLES/GM FRESH WT			
		MG N/GM FRESH WT	% OF TOTAL HOMOGENATE	TOTAL	NON-PROTEIN	MICROMOLES/MG N × 10^2	% OF HOMOGENATE
Homogenate	.....	6.95	100	3.95	0.25	57	100.0
Starch	1,000 *	0.28	4	0.07	0.01	25	1.8
Chloroplast	4,000 **	0.16	2	0.05	0.02	31	1.2
Mitochondria-chloroplast	500,000 †	1.18	17	0.26	0.07	22	6.6
Soluble	500,000 †	5.55	80	1.90	0.21	34	48.1
Recovery	.....	...	103	...	...	..	57.7

Fractions prepared from sucrose-phosphate-magnesium chloride homogenates of mature leaves of *Phaseolus vulgaris*. -SH determined by titration in alcohol ammonia.
* 1000 × g for 1 min.
** 1000 × g for 4 min.
† 16,700 × g for 30 min.

lected at about 1000 g-minutes; a small fraction of relatively pure chloroplasts were sedimented at 4000 g-minutes; and a mixture of mitochondria and the remaining chloroplasts obtained at 500,000 g-minutes. The sulfhydryl and nitrogen data are summarized in table III. In bean leaves the "soluble" fraction again had the bulk of the protein sulfhydryl, but more than 10 % of the recovered non-protein sulfhydryl appeared in the mitochondria-chloroplast fraction.

From tables II and III it may be calculated that the sum of the total protein -SH contents of the several fractions is considerably less than that of the homogenate, whereas the non-protein -SH nearly coincides. Why the protein -SH should be specifically affected is not known. It is possible that the particulate component became susceptible to autoxidation during washing.

In both tissue systems described the cell debris was neglected because of the technical problem of keeping the platinum electrode free of fiber. A few titrations of preparations of washed fiber were successful, and in the case of pea seedlings a figure of about 1 % of the homogenate -SH was found.

DISCUSSION

The inherent errors as well as the advantages of the amperometric methods for sulfhydryl analysis have been discussed in detail by Benesch et al (3). Application of the method to certain plant material has presented only one difficulty, the relatively rapid decrease in -SH content after grinding. Losses in the pea stem between grinding and titration were estimated at 12 %. It would be advantageous to have present in the homogenizing medium a reagent to mask the -SH groups, as is employed in the chromatographic separation of non-protein sulfhydryls (18). Recent experiments indicate that the amperometric method can be modified to include this precaution by homogenizing the sample in an estimated 10 % *excess* of silver nitrate in TRIS buffer and back-titrating with standardized glutathione.

The application of amperometric titration to plant tissues permitted certain preliminary observations. First, the -SH contents of the four plant tissues examined varied over a 50-fold range (table I and III). The physiological consequences of these differences may prove of importance. Second, comparisons can be made with measurements in the animal literature. Flesch and Kun (6) assembled data [5] for various mammalian tissues for which the -SH contents were typically 1 to 2 micromoles per gram; liver, for example, was reported as containing 1.5 micromoles per gram. Plant tissues appear to be in the same order of magnitude: nearly 2 micromoles per gram for pea seedlings (table II) and nearly 5 micromoles per gram for bean leaves (table III). Ehrenberg (5), also employing silver titration, found about 2 micromoles -SH per gram in barley embryos.

About 90 % of the plant tissue sulfhydryl can be attributed to proteins, a proportion very similar to that in red blood cells (2). The values for micromoles -SH per mg protein N for plant tissues are as follows: 0.16 for pea seedlings, 0.18 for oat coleoptiles, 0.45 for pea stems, 0.57 for bean leaves, and up to 0.8 for lily anthers (23). The corresponding values for crystallized enzymes were found by Benesch et al (23) to range from 5 to 12.

The employment of the amperometric method of sulfhydryl estimation in growth studies is reported in the following contribution (19).

SUMMARY

1. Amperometric silver titration was employed for the estimation of the sulfhydryl contents of etiolated

[5] There is a discrepancy between these figures and those of Benesch and Benesch (1). The latter's measurements of glutathione exceed the *total* -SH levels reported by Flesch and Kun.

pea seedlings, oat coleoptiles, bean leaves, and sunflower internodes.

2. Preliminary estimates of the protein and nonprotein sulfhydryl contents of sub-cellular fractions are also reported. The -SH : protein N ratios were fairly uniform among the fractions.

3. Plant sulfhydryl contents per unit of fresh weight were found to compare closely with values reported for mammalian tissue.

The authors are greatly indebted to Dr. A. C. Leopold for his encouragement and helpful criticisms.

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THE EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON THE RESPIRATION OF ETIOLATED PEA SEEDLINGS[1,2]

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Since the report of Brown (6) that 2,4-dichlorophenoxyacetic acid (2,4-D) increased the rate of carbon dioxide production from intact bean plants, many reports on the influence of 2,4-D on respiration have appeared in the literature. Smith (14) and Avery (1) have reviewed the literature which appeared before 1950. Some of the more recent papers are listed in the "Literature Cited" section of this paper (7, 8, 12, 17, 18).

French and Beevers (7) suggest that the increase in respiration induced by 2,4-D, indoleacetic acid (IAA), and other plant growth substances is a result of the increase in growth induced by these substances. An increase in growth with an attendant increase in anabolic reactions would increase the supply of high energy phosphate acceptors, and thus, presumably, increase the rate of respiration. This suggestion was based, in part, on the findings that these plant growth substances, in the concentration range that increased the elongation of corn coleoptile segments, increased the respiration of the segments, while 2,4-dinitrophenol, which inhibited growth, also caused a marked increase in the rate of respiration. The work of Kelly and Avery (12) lends support to this interpretation. These workers found that, although the respiration of young pea stem tissue was markedly stimulated by 2,4-D, the respiration of tissue slices from older pea stem segments which had ceased to elongate was not increased by 2,4-D. Bonner and Bandurski (4), in their discussion of the action of IAA on respiration and growth, are inclined to a view similar to that of French and Beevers (7).

This paper reports the results of an investigation on the phenomenon of respiratory stimulation by 2,4-D with respect to the qualitative and quantitative changes in the respiratory substrate. It is postulated that 2,4-D increases respiration by causing more glucose to be catabolized via the pentose phosphate pathway.

A preliminary note concerning part of this work has been published (9).

MATERIALS AND METHODS

Dry pea seeds (*Pisum sativum*, var. Emerald) were soaked overnight in tap water with aeration, planted in moist sand, and placed in the dark at 20° C for 60 hours. During this time the seedlings reached a length of 3 to 5 cm, most of which was unbranched, primary root.

Corn seeds (*Zea Mays*, var. Funks G-50) were soaked overnight with aeration and placed in Pyrex trays on moist filter paper. The trays, covered with a sheet of glass, were placed in the dark at 20° C for 60 hours.

The seedlings were washed in distilled water and placed in vials containing either 10^{-2} M potassium phosphate buffer, pH 5.3, or buffer plus 10^{-3} M 2,4-D. Only the distal 1 to 2 cm of the root were immersed in the solution. The vials were placed in the dark at 20 to 21° C for 12 hours. Seedlings were treated both with and without the cotyledons attached. In some instances treatment was done in the Warburg vessels by tipping a buffered 2,4-D solution from the side arm into the main compartment after the first hour of manometric measurement. For these latter experiments only seedlings from which the cotyledons had been removed were used.

2,4-D (Eastman Kodak Co.) was neutralized with NaOH, and the sodium salt recrystallized two times from a water-alcohol solution.

RESPIRATION MEASUREMENTS: After the 12-hour treatment period, the seedlings were removed from the vials, washed in distilled water, blotted on filter paper and weighed to the nearest milligram in groups of three. Three seedlings weighed from 0.3 to 0.4 gm. The weights of the 2,4-D-treated seedlings were not significantly different from the weights of the control seedlings. For the measurement of gas exchange the seedlings were inserted upright into standard 15-ml conical Warburg vessels which contained enough potassium phosphate buffer (10^{-2} M, pH 5.3) to cover approximately one cm of the root tip. Seedlings from which the cotyledons had been removed were used in all cases for the respiration measurements. The cotyledons were removed either just before the 2,4-D treatment or after the treatment and before weighing.

In some experiments the respiration of pea root tips was measured. Root tips, approximately 2 cm long, were cut from the pea seedlings which had been treated as above with 2,4-D or buffer. The root tips were weighed in groups of seven (each group weighed about 0.2 gm) and placed in Warburg vessels containing 2.8 ml of potassium phosphate buffer (10^{-2} M, pH 5.3).

The manometric experiments were conducted in the Warburg apparatus at 25° C. The vessels were shaken in the dark. For the measurement of oxygen uptake, the vessels were filled with air or oxygen and 0.2 ml of 20 % KOH was added to the center well. The "direct method" (16) for the measurement of carbon dioxide evolution was used.

In a number of experiments the seedlings or root tips were removed from the Warburg vessels at the end of the experimental period, and the buffer solutions remaining in the vessels were tested for oxygen

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uptake. It was concluded that bacterial or fungal contamination was not significant at the concentration of 2,4-D used.

Q_{O_2} as used in this paper, is defined as μl of oxygen utilized per hour per gm fresh weight of tissue.

CARBOHYDRATE ANALYSIS: Seedlings were killed with hot 90 % ethanol, and then extracted with 80 % ethanol for 16 hours. The concentration of reducing sugars in the extract was estimated using the method of Nelson (13). Sucrose was estimated by analyzing the extract for reducing sugars before and after invertase treatment. For the determination of starch the alcohol insoluble residue was dried at 70° C and ground in a mortar with water, and the resulting suspension was boiled for two minutes to solubilize the starch. The starch was hydrolyzed with takadiastase followed by 2.5 % HCl. The hydrolyzed starch solution was neutralized with KOH, made up to volume and analyzed for reducing sugars.

RESULTS

EFFECT OF 2,4-D ON OXYGEN UPTAKE: In table I are shown the results of a typical experiment in which 2,4-D in several concentrations was added to the Warburg vessels containing the pea seedlings, and respiration measurements were begun immediately. This is in contrast to later experiments in which the pea seedlings were pre-treated with 2,4-D followed by respiration measurements in the absence of 2,4-D. The greatest increase in oxygen uptake above the controls (after 18 hours) was shown by seedlings treated with 10^{-3} M 2,4-D. Higher concentrations of 2,4-D (10^{-2} M) caused breakdown of the root tissue, and bacterial or fungal contamination set in after 6 to 8 hours. In view of these results, 10^{-3} M 2,4-D was used in all subsequent experiments.

Pea seedlings were pre-treated with 2,4-D or buffer, both with and without the cotyledons attached. In table II are shown results of an experiment in which pea seedlings were pre-treated after the cotyledons had been removed. There was very little difference between the Q_{O_2} values of 2,4-D-treated and control seedlings during the first 3 hours after treatment. However, a large difference in Q_{O_2} values was obtained 9 hours after treatment. Note that when seedlings were treated with 10^{-3} M 2,4-D in the Warburg vessel a large increase in Q_{O_2} over the controls resulted after 18 hours (table I). The results in table II show that 3 hours after treatment (15 hours after the initial contact with 2,4-D) there was very little difference in Q_{O_2} between the 2,4-D and buffer pre-treated seedlings. These differences in rate of response to 2,4-D are most probably attributable to differences in the temperature during treatment. Seedlings treated in the Warburg vessel were at 25° C; pre-treated seedlings were maintained at 20° C during treatment and at 25° C during respiration measurements.

The differences in Q_{O_2} shown in tables I and II did not result from 2,4-D increasing the initial respiration rate, but rather were caused by a difference in the rates at which the respiration of the 2,4-D treated

TABLE I

EFFECT OF 2,4-D ON THE OXYGEN UPTAKE OF PEA SEEDLINGS

TREATMENT *	Q_{O_2} (WET WT)		
	FIRST 150 MIN	AFTER 18 HRS	% OF CONTROL AFTER 18 HRS
Buffer	455	157	...
10^{-3} M 2,4-D + Buffer	466	298	190
10^{-4} M 2,4-D + Buffer	471	229	146
10^{-5} M 2,4-D + Buffer	466	225	143
10^{-6} M 2,4-D + Buffer	474	179	114

* Etiolated pea seedlings, after removing the cotyledons, were weighed and immediately placed in Warburg vessels (3 seedlings per vessel) containing either 10^{-2} M phosphate buffer, pH 5.3, or buffer plus 2,4-D.

TABLE II

EFFECT OF 12-HOUR PRE-TREATMENT WITH 10^{-3} M 2,4-D ON THE OXYGEN CONSUMPTION OF PEA SEEDLINGS

TREATMENT *	Q_{O_2} (WET WT)	
	FIRST 3 HRS AFTER TREATMENT	9 HRS AFTER TREATMENT
Buffer	341	188
10^{-3} M 2,4-D + Buffer	352	314

* Etiolated pea seedlings, after removing the cotyledons, were placed in the dark in either 10^{-2} M phosphate buffer, pH 5.3, or buffer plus 10^{-3} M 2,4-D. Seedlings were removed from treating solutions after 12 hours, washed, weighed and placed in Warburg vessels with 10^{-2} M phosphate buffer, pH 5.3.

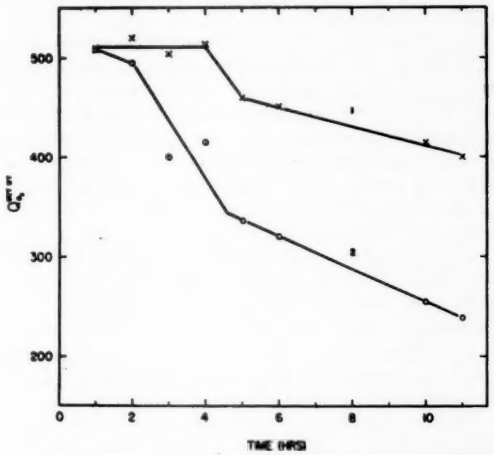

FIG. 1. Effect of 2,4-D treatment of pea seedlings with cotyledons attached on the subsequent respiration rates after removing the cotyledons. Curve 1. Seedlings pre-treated for 12 hours at 20° C with 10^{-2} M phosphate buffer, pH 5.3, plus 10^{-3} M 2,4-D. Curve 2. Seedlings pre-treated with buffer alone.

and control seedlings declined as the seedlings used up their food reserves. 2,4-D did not affect the respiration rate of pea seedlings with high levels of food reserves. The results of another experiment which are plotted in figure 1 show this more clearly. In this experiment the seedlings were pre-treated for 12 hours with 10^{-3} M 2,4-D as before, but the cotyledons were left on, insuring an adequate food supply during the treatment period. The cotyledons were removed just before weighing the seedlings preparatory to placing them in the Warburg vessels. At the beginning of the respiration measurements, the Q_{O_2} was the same for both the 2,4-D-treated and untreated seedlings; only when the Q_{O_2} started to decrease did differences in respiration rate occur. Similar results were obtained with seedlings pre-treated with 2,4-D for only 6 hours showing that the lack of respiratory response to 2,4-D by the intact seedlings was a result of the presence of the cotyledons, and was not due to the length of time necessary for the penetration, translocation and action of the herbicide. Furthermore, seedlings pre-treated for 12 hours at 25° C (instead of 20° C) showed a similar response to 2,4-D. Thus the change from the pre-treatment temperature to the temperature at which the Q_{O_2} was measured did not account for the results shown in figure 1.

In the previous experiments respiration measurements were made on seedlings which were in an air atmosphere. The effect of an oxygen atmosphere on the Q_{O_2} of pea seedlings (2,4-D-treated and untreated) was tested. The results obtained were quite similar to those shown in figure 1; the Q_{O_2} values in oxygen being almost identical with the values obtained in air. This was true for both the 2,4-D-treated and untreated seedlings. The results obtained in previous experiments, then, were not complicated by an oxygen limitation.

Corn seedlings were used to investigate the effect of 2,4-D on the respiration of monocots. The seedlings (endosperm attached) were pre-treated with 10^{-3} M 2,4-D in the same manner as were the pea seedlings. The endosperm and scutellum were removed before the corn seedlings were placed in the Warburg vessels. The results were quite similar to those shown in figure 1 for pea seedlings. Thus, the respiratory response to 2,4-D was, under the particular experimental conditions used, essentially the same for intact seedlings of both monocots and dicots. Kelly and Avery (11) found that pea stem tissue responded to lower concentrations of 2,4-D than did oat coleoptile tissue. No attempt was made in this study to compare the respiratory sensitivity to 2,4-D of intact seedlings of monocots and dicots.

Kelly and Avery (11, 12) found that 2,4-D brought about a greater respiratory stimulation in starved oat coleoptile and pea stem slices than in unstarved slices. The results of the experiments presented in this paper show that the respiration of entire pea seedlings was not influenced by 2,4-D when the seedlings had a high level of food reserves (cotyledons attached). Furthermore, the longer the seedlings were starved after removing the cotyledons and before 2,4-D treatment the more quickly their respiration responded to 2,4-D. These results are shown in table III. It should be noted that starvation affected only the time necessary for the response to begin; the magnitude of response, measured as percent of control, was not appreciably changed in ten hours. The effect of the cotyledons or of starving on the response of the seedling respiration to 2,4-D was mentioned above as being due to "high" or "low" food reserves. Kelly and Avery (11) have shown that the respiration of oat coleoptile tissue was stimulated to a greater extent by 2,4-D when the tissue was in water rather than in sucrose. It is recognized, nevertheless, that with intact pea seedlings other substances, not foodstuffs, moving from the cotyledons to the root-shoot axis may be modifying the action of 2,4-D.

2,4-D AND THE RESPIRATORY SUBSTRATE: Since seedlings treated with 2,4-D were able to maintain a higher rate of respiration than untreated seedlings under conditions of declining respiration rate due to depletion of food reserves, the question arises as to

TABLE III

EFFECT OF STARVING ON THE RESPONSE OF SEEDLING RESPIRATION TO 2,4-D

HRS AFTER TIPPING 2,4-D **	LENGTH OF STARVING PERIOD *		
	1 HR	13 HRS	19 HRS
	% Q_{O_2} of control		
2	100	100	100
3	103	114	116
5	114	122	136
10	137	144	...

* Starving period. Seedlings, after removing the cotyledons, were placed in the dark at 20° C for the periods shown. Manometric measurements made at 25° C.

** At the end of the vessel equilibrium period a buffered 2,4-D solution (pH 5.3) was tipped from the side arm. Final concentration of 2,4-D in main compartment, 10^{-3} M.

TABLE IV

EFFECT OF 12-HOUR PRE-TREATMENT WITH 10^{-3} M 2,4-D ON THE R.Q. OF PEA SEEDLINGS

TREATMENT *	Q_{O_2}	Q_{CO_2}	R.Q.	Q_{O_2}	Q_{CO_2}	R.Q.
	FIRST 4 HRS AFTER TREATMENT			9TH AND 10TH HRS AFTER TREATMENT		
Buffer	431	412	0.96	249	239	0.96
	431	404	0.94	249	233	0.94
10^{-3} M 2,4-D + Buffer	476	471	0.99	373	369	0.99
	476	476	1.00	373	369	0.99

* Etiolated pea seedlings, cotyledons attached, were placed with their roots in either 10^{-2} M phosphate buffer, pH 5.3, or buffer plus 10^{-3} M 2,4-D. Treatment was for 12 hours in the dark at 20 to 21° C. At the end of the treatment period the cotyledons were removed and the seedlings were washed, weighed and placed in Warburg vessels with 10^{-2} M phosphate buffer, pH 5.3.

what kind of food reserves were being used as respiratory substrates in each case. Two lines of evidence point to carbohydrate as being the major respiratory substrate. First, the respiratory quotients (R.Q.) of the 2,4-D-treated and control seedlings were nearly the same and approximated one (table IV). These results were obtained both during the first four hours after treatment when the difference in Q_{O_2} between the 2,4-D-treated and untreated seedlings was small, and during the ninth and tenth hours after treatment when the difference in Q_{O_2} was large (50%). The second line of evidence which points to carbohydrate as the major respiratory substrate is that there was a proportionality between the decrease in reducing sugar and the decrease in Q_{O_2} of the seedlings. Approximately 200 seedlings (minus cotyledons) with their roots in buffer were placed in the dark at 25° C. At intervals over a 27-hour span, seedling samples were removed for Q_{O_2} determination and carbohydrate analysis. From the results shown in figure 2 it can be seen that the decrease in reducing sugar and in starch paralleled the decrease in Q_{O_2}. The small amounts of sucrose found (0.2 to 0.3 mg per gm fresh weight) did not vary with time, and are not included in the graph. Assuming $Q_{O_2} = k$ [reducing sugar], k was calculated from the reducing sugar and Q_{O_2} values at different times over the 27-hour period of measurement. The values of k ranged from 30 to 37 μl per mg hr with an average of 34.

CARBOHYDRATE ANALYSES: The difference in rate of respiration between 2,4-D-treated and untreated pea seedlings apparently was not due to a qualitative difference in respiratory substrate. It may, however, have been due to a quantitative difference, and, since carbohydrate was indicated as the major respiratory substrate, the reducing sugar, sucrose and starch contents of the seedlings were determined at different times during the experimental period.

If 2,4-D increased the amount of carbohydrate translocated from the cotyledons to the root-shoot axis, then intact seedlings pre-treated with 2,4-D would be able to maintain a higher rate of respiration than untreated seedlings after the cotyledons were removed. Such a possibility was investigated with the results shown in table V, experiments 1 and 2. In these experiments pea seedlings with the cotyledons attached were treated with 2,4-D or buffer for 12 hours at 20° C. At the end of this period the cotyledons were removed and the root-shoot axis taken for carbohydrate analysis. At the time the seedlings were killed for analysis, there was no difference in the Q_{O_2} values between the 2,4-D-treated and control seedlings as shown in figure 1 (Q_{O_2} values for the first hour). There was also very little difference in carbohydrate content of the seedlings at this time (table V, experiments 1 and 2). 2,4-D, then, does not have any effect on the carbohydrate content of seedlings during the pretreatment period.

What, however, is the carbohydrate picture when the 2,4-D-treated seedlings are respiring at a much higher rate than the control seedlings? To answer this question the following experiment was run. Pea

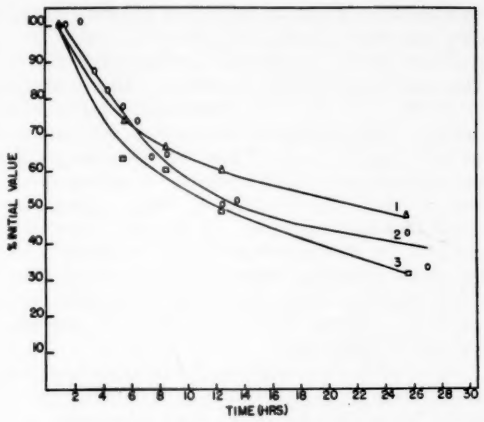

FIG. 2. Decrease in the reducing sugar content (curve 1), starch content (curve 3), and respiration rate (curve 2) of pea seedlings with time after removing the cotyledons.

TABLE V

EFFECT OF 2,4-D ON THE CARBOHYDRATE CONTENT OF PEA SEEDLINGS

EXPT NO.	TREATMENT *	REDUCING SUGAR MG/GM	SUCROSE MG/GM	STARCH (AS GLUCOSE) MG/GM	TOTALS MG/GM
1	Buffer (+ cotyledons)	11.1, 10.6	0, 0.2	...	...
	2,4-D (+ cotyledons)	10.8, 11.7	0.2, 0.3	...	...
2	Buffer (+ cotyledons)	11.0, 10.6	0,	2,0, 2.2	12.9
	2,4-D (+ cotyledons)	11.8, 11.5	0.6, 0.7	2.2, 2.2	14.6
3	Buffer (starved)	8.0, 7.4	2.7, 2.2	0.64, 0.84	10.9
	2,4-D (starved)	8.0, 8.0	2.5, 3.1	0.57, 0.63	11.4

* Experiments 1 and 2. Seedlings, cotyledons attached, were treated with 10^{-3} M 2,4-D plus 10^{-2} M phosphate buffer (pH 5.3) or with buffer alone for 12 hours in the dark at 20° C. At the end of this period the cotyledons were removed and root-shoot axes were analyzed for carbohydrate.

Experiment 3. Pea seedlings, after removing the cotyledons, were treated with 10^{-3} M 2,4-D plus buffer or with buffer alone for 12 hours in the dark at 20° C. At the end of this period all seedlings were transferred to buffer and placed in the dark at 25° C for 7 hours; after which period the seedlings were removed for carbohydrate analysis.

seedlings from which the cotyledons had been removed were pre-treated with 2,4-D or with buffer for 12 hours at 20° C. At the end of this period all seedlings were placed in buffer at 25° C. After 7 hours at 25° C the seedlings were removed for carbohydrate analysis. The results of this analysis are shown in table V, experiment 3. There was very little difference between the 2,4-D-treated and untreated seedlings in the levels of reducing sugars, sucrose and starch that they contained. In separate experiments in which the seedlings were treated in identical fashion except that Q_{O_2} values were determined instead of carbohydrate levels, the 2,4-D-treated plants were respiring at a rate 50 to 60 % above the controls 7 hours after treatment.

Since after treatment the 2,4-D-treated seedlings are respiring at a higher rate, it would be expected that 7 hours after treatment the 2,4-D-treated seedlings would contain less carbohydrate than the control seedlings. Although the 2,4-D-treated seedlings were respiring at rates 50 to 60 % above the controls 7 hours after treatment, the sum total difference in oxygen uptake over this period was of the order of 450 μl per gm fresh weight. The oxidation of one mg of glucose to CO_2 and water requires 744 μl of oxygen. Thus a difference of 450 μl would require only 0.6 mg of glucose. Such a small quantity is within the limits of variability of the samples in a single treatment (see table V, expt. 3).

Discussion

From the results presented in this paper it seems unlikely that the increased respiration accompanying 2,4-D treatment was due to a qualitative or a quantitative change in the respiratory substrate. This was true despite the fact that the rate of respiration was limited by the concentration of respiratory substrate. How, then, is 2,4-D acting in stimulating respiration? It has been suggested that 2,4-D and auxins increase the amount of high-energy phosphate acceptors either by increasing anabolic reactions (4, 7) or by uncoupling oxidative phosphorylations (5, 15). If this is true with pea seedlings, then one would expect 2,4-D to increase the Q_{O_2} of seedlings high in carbohydrates such as those treated with the cotyledons attached. This was not the case (see fig 1, Q_{O_2} for first hour). Furthermore, although no growth measurements were made on the pea seedlings, it was evident from a cursory examination that 10^{-3} M 2,4-D inhibited the growth in length of the roots during the 12-hour pretreatment period. An increase in growth due to 2,4-D and auxin was the basis for supposing that these substances caused an increase in anabolic reactions and thus an increase in high-energy phosphate acceptors (7). French and Beevers (7) found that the growth and respiration of corn coleoptile segments responded similarly to various concentrations of 2,4-D. Concentrations of 2,4-D which inhibited growth also inhibited respiration. This was not the case with intact pea seedlings.

In this laboratory it was recently found that in root tips 2,4-D caused an increase in the amount of glucose catabolised via the pentose-phosphate pathway (10). The amount of glucose catabolised via the Embden-Meyerhof glycolytic pathway was unchanged in root tips of corn seedlings and in root tips of pea seedlings from which the cotyledons were removed before 2,4-D treatment. However, in root tips from pea seedlings 2,4-D-treated with the cotyledons attached less glucose was catabolised via the glycolytic pathway than in the controls. These results were obtained by using the method of Bloom and Stetten (3) in which the initial yields of $C^{14}O_2$ from glucose-1-C^{14} (G-1-C^{14}) and glucose-6-C^{14} (G-6-C^{14}) are used to estimate the degree of participation of the two pathways.

Since root tips were used in studying the effect of 2,4-D on pathways of glucose catabolism, it became necessary, if a correlation between the respiratory response to 2,4-D and the effect of 2,4-D on pathways of glucose catabolism was to be attempted, to study the effect of 2,4-D on the respiration of root tips. In these experiments root tips from 2,4-D-treated and untreated pea and corn seedlings were used. The pea seedlings were pre-treated with 2,4-D both with and without the cotyledons attached; corn seedlings were treated with the endosperm attached. The results obtained with root tips of pea seedlings are shown in figure 3. The upper set of curves was obtained using root tips from seedlings from which the cotyledons

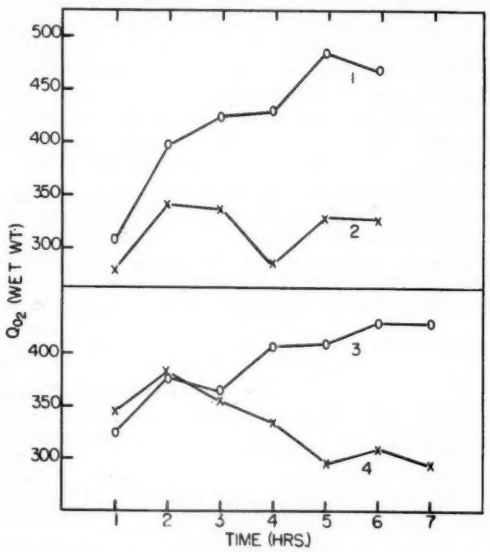

Fig. 3. Respiration rates of pea root tips from 2,4-D-treated and untreated seedlings. Curves 1 and 2. Root tips from seedlings from which the cotyledons were removed prior to treatment with 2,4-D (10^{-4} M) plus buffer (curve 1) or buffer alone (curve 2). Curves 3 and 4. Root tips from seedlings with cotyledons attached during treatment with 2,4-D (10^{-4} M) plus buffer (curve 3) or buffer alone (curve 4).

were removed prior to treatment. The respiration rate of root tips from the 2,4-D-treated seedlings (curve 1) became progressively greater, from the first hour, than the rate exhibited by root tips from untreated seedlings (curve 2). The lower set of curves was obtained using root tips from seedlings treated with the cotyledons attached. With root tips from these latter seedlings 2,4-D had no effect on the respiration rate for the first three hours. After three hours root tips from 2,4-D-treated seedlings (curve 3) respired at a progressively greater rate while the respiration rate of root tips from untreated seedlings (curve 4) declined.

Root tips from corn seedlings, 2,4-D treated with the endosperm attached, showed the same respiratory response as did root tips from pea seedlings treated with the cotyledons removed (curves 1 and 2, fig 3).

The respiratory response of pea root tips to 2,4-D showed some variability, and exactly the same results as those shown in figure 3 were not always obtained. In some experiments the break in the curves (curves 3 and 4) occurred sooner than three hours, and in some cases curves 1 and 2 did not separate until the second hour.

Because only the initial or short-time yields of $C^{14}O_2$ from G-1-C^{14} and G-6-C^{14} are meaningful in the analysis of catabolic pathways (2), this type of experiment was run for only 3.5 hours (10). Therefore, when attempting to correlate the effect of 2,4-D on respiration with its effect on pathways of glucose catabolism, only the results obtained during the first 3.5 hours should be taken into account. If this is done, it can be seen that each respiratory response or lack of response to 2,4-D finds its counterpart in the changes in pathways of glucose catabolism due to 2,4-D. Corn root tips and root tips from pea seedlings from which the cotyledons were removed before 2,4-D treatment showed an immediate increase in respiration rate over the controls; in these root tips more glucose was catabolised via the pentose phosphate pathway while the amount going via the glycolytic pathway was unchanged (10). Root tips from pea seedlings which had their cotyledons attached during 2,4-D treatment showed very little increase in total oxygen uptake over the control seedlings during the first 3.5 hours; in these root tips more glucose was catabolised via the pentose phosphate pathway, but, counterbalancing this, less glucose was catabolised via the glycolytic pathway (10).

The results presented in this and a previous report (10) suggest, at least with root tips, that the stimulation of respiration elicited by 2,4-D is a result of the action of 2,4-D in shunting more glucose through the pentose phosphate pathway. Since intact seedlings do not lend themselves to experiments in which rates of $C^{14}O_2$ production from added labeled compounds are measured, an estimation of the participation of the pentose phosphate pathway in the intact seedling was not possible. Nevertheless, it is attractive to postulate that the above explanation also holds for the intact seedling. A note of caution is in order, however, since the absolute respiration rates of the 2,4-D-treated seedlings declined with time while the absolute respiration rates of the root tips obtained from such seedlings increased with time (compare figs 1 and 3).

Further insight into this problem awaits an answer to the question: How and where does 2,4-D act to cause more glucose to be catabolised via the pentose phosphate pathway?

Summary

1. The respiration rate of etiolated pea seedlings declined rapidly after removal of the cotyledons. 2,4-D-treated pea seedlings were able to maintain a higher rate of respiration during this period of decline than were untreated seedlings.

2. R.Q. data indicate that both the 2,4-D-treated and untreated seedlings utilize carbohydrates as the primary respiratory substrate.

3. The reducing sugar, sucrose and starch content of the 2,4-D-treated and untreated seedlings were essentially the same.

4. It is concluded that the higher rate of respiration exhibited by the 2,4-D treated seedlings is not due to a greater amount of respiratory substrate being present in these seedlings.

5. Respiratory data obtained from root tips of 2,4-D-treated and untreated pea and corn seedlings are presented, and are compared with data from a previous report (10) concerning the effect of 2,4-D on pathways of glucose catabolism in root tips.

6. From the data presented, it is postulated that 2,4-D increases respiration by causing more glucose to be catabolised via the pentose phosphate pathway.

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EFFECTS OF OXYGEN AND RED LIGHT UPON THE ABSORPTION OF VISIBLE LIGHT IN GREEN PLANTS[1,2]

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In studies of the purple bacteria, the correlation between the effects of oxygen and light provide direct information on the nature of the light reaction and assist in identifying the point in the respiratory chain at which oxidants produced in the photochemical process react (1). In addition, a comparison of the effects of respiratory inhibitors upon the absorption spectra of the pigments of the purple bacteria provide further bases for analysis of light effects (1, 2). Although spectroscopic changes due to illumination of Chlorella and other green cells have been reported from several laboratories (3 to 10), in which some studies of inhibitors and activators of the effects have been made, no dark process has been described which produces effects similar to illumination. We find that the spectra representing the differences of absorption between the aerobic and anaerobic green cells show great similarity to those representing the differences between dimly-lighted and dark cells. Such data have been obtained for two types of green algae. In brief, our data show that oxygenation or illumination of the anaerobic cells causes an absorption band to appear at 518 mμ and one to disappear at 475 mμ. Since our method is suitable for the recording of slow changes of absorption as well as rapid ones, the time course of the light and dark reactions has been studied. Three distinct light reactions are described. One reaction is the small and rapid "aerobic light effect" (phase 2) studied by previous workers (3 to 10) and a second one is observed with dim illumination of anaerobic cells (phase 3) that is related to oxygenation of the cells. The third (phase 1) is the "recovery phase" of an overshoot phenomenon (cf. 6). The nature of these reactions and their relation to the photosynthetic process is discussed. A brief report on part of this work has appeared (19).

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Methods

Methods for measuring spectroscopic changes in photosynthetic cells are reviewed by Duysens (17) who has done pioneer work in this field. Our spectroscopic method has previously been used in photochemical studies (12) but the sample holder is similar to Witt's (7).

Figure 1 shows the apparatus especially designed for dense algal suspensions or sections of leaves. The moist chamber, which is not visible in figure 1, has a volume of 1.2 ml ($20 \times 20 \times 30$ mm). About 0.7 ml of an algal suspension containing 0.1 to 0.2 ml of cells is added and the algae are allowed to settle on the transparent base of the chamber and form a uniform layer suitable for spectrophotometric observation. The moist chamber fits into a bakelite plate which mounts directly on top of an end-on photomultiplier (fig 1). Between the algae and the photosurface is interposed a suitable filter, e.g., Wratten #44 A or Corning 5030. Moist gases containing proper proportions of nitrogen, oxygen and carbon dioxide are passed over the

surface of the liquid in the moist chamber via the connections indicated in figure 1. In order to monitor the oxygen tension and to test the photosynthetic activity of the cells, two types of platinum micro-electrodes are used. First, a micro-electrode and its associated silver reference electrode project into the base of the moist chamber and record, through suitable amplifiers, changes in oxygen concentration that occur upon oxygenation of the cells by illumination or by gas mixtures. Secondly, for more sensitive measurements, a spiral platinum wire imbedded in the lucite base of the chamber is used with reference to a calomel electrode (11).

The light for activating the photochemical systems is obtained in two ways: a) six small light bulbs are mounted directly before the 45° mirror and are painted with red nail polish which excludes, to a considerable extent, wavelengths shorter than 640 $m\mu$; b) the 45° mirror is partly silvered so that illumination from above by means of a 100 watt tungsten lamp and lens combination can be used with Corning 2403 filter. About one third of the exciting light is transmitted by the 45° mirror. The photomultiplier is protected from this red light in two ways: 1) by the chlorophyll present in the suspension studied and 2) by the filter interposed between the cell suspension and the photomultiplier. In addition, the exciting light is steady while the measuring light is flickered. Thus, light-leaks onto the phototube increase the steady component of the photocurrent and hence the noise output, but this does not cause any net displacement of the recorders responsive to the measuring signal. A full discussion of the application of these methods to photochemical reactions is given elsewhere (12, 13).

The spectrophotometer is of the double beam bichromatic type (14) and the two monochromators can be seen at the rear of figure 1. The monochromatic beams, for example 515 and 495 $m\mu$, emerging from these monochromators fall upon a 60-cycle vibrating mirror which alternately flickers light of the two wavelengths upon the 45° mirror and thence through the material under observation. Since capacitance-coupled amplifiers are used to amplify the difference in the phototube response to the two flashes of light, the output is unresponsive to the exciting light, provided it does not give signals which exceed the linear range of the amplifying circuits. Suitable controls on the freedom from artifact due to the exciting light are obtained either by turning on the exciting light in the absence of the measuring light or, alternatively, setting the wavelength of the measuring beams to the same value and then turning on the exciting light. In both cases, negligible deflection of the output of the

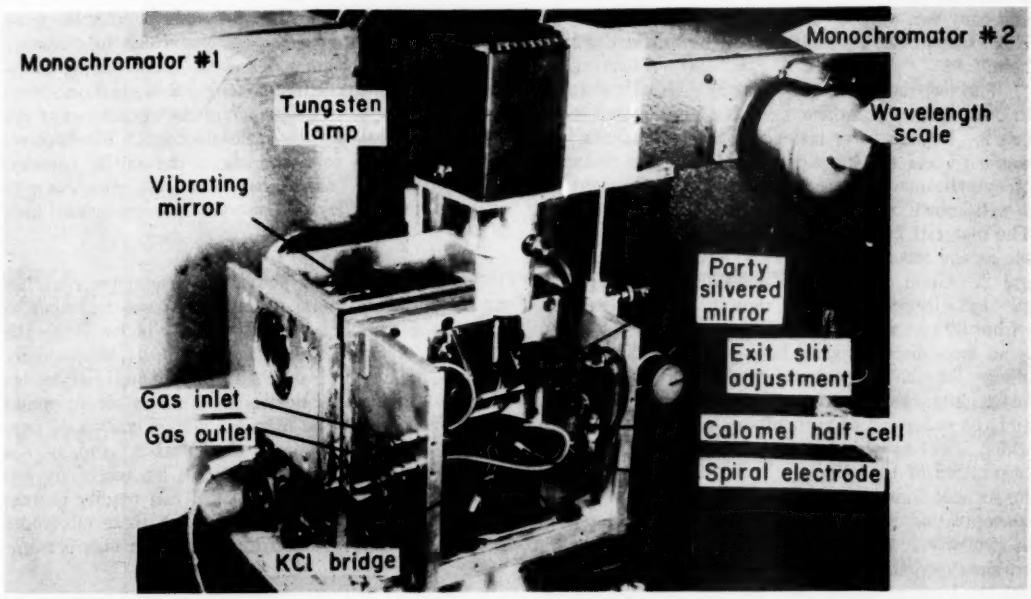

FIG. 1. A photograph of the moist chamber for spectroscopic measurements of the effects of illumination upon the green plant cells. The illustration shows in the background the monochromators of the double beam spectrophotometer, the image of the exit slits which are focused on a vibrating mirror enclosed in the box in the background. The light is reflected from the vibrating mirror onto the partly silvered 45° mirror and then downward onto the 1.5-cm^2 moist chamber housed in a bakelite disk and mounted upon the magnetic shield for the end-on photomultiplier. A 100-watt lamp and lens combination illuminates the algae by the transmission through the partly silvered mirror. The gas connection for oxygenating or disoxygenating the material in the moist chamber is shown. The connections for the platinum micro-electrode and its silver reference electrode are also shown. A cover encloses the assembly to avoid stray light. (FA 35).

recorders occurs under the conditions used in these experiments (12, 13).

Spectroscopic measurements are usually made with respect to a neutral wavelength at which no absorption changes have been found to occur. For the conditions of our experiment, 495 mμ is used as a reference wavelength. The measuring wavelength can be adjusted as desired in order to record the light absorption changes with respect to the reference wavelength. The sensitivity of the apparatus normally used is about 1 % change of absorption for full scale of the recorder. The noise level is very near the theoretical value and the records included here show fluctuations less than 10^{-4} in optical density with samples of adequate transparency. The spectral interval used is less than 3 mμ and is often 1.5 mμ. This double beam method gives a clearer result than that obtained by the compensating methods used by Duysens (3) and this is best summarized by a comparison of original experimental records shown here and in reference 17.

Kok (10) has recently had some success with a method of electrical sampling or "gating" of the photocurrent at arbitrary times after the flash. Such a method is suitable for accurate measurements at these arbitrary times, which must be chosen on the basis of the entire time course of the reaction kinetics. The instrument does not, however, plot out such kinetics and they can only be obtained with difficulty. Thus Kok's instrument is unsuitable for the studies described here.

The response of the material in the moist chamber to oxygenation and disoxygenation is indicated in figure 2. In this case, instead of algae, Baker's yeast was used and the kinetics of oxidation and reduction of cytochrome c are recorded, since this pigment has a well-known response to changes of oxygen tension. The material in the moist chamber is initially anaerobic as the record begins on the left-hand edge of figure 2. Upon admission of oxygen, the oxidation of the cytochrome proceeds rapidly and is complete within 60 seconds. Upon admission of nitrogen, there is no immediate response because of the high oxygen affinity of the cytochrome system. However, after one minute, the oxygen tension reaches a critical value and the reduction of cytochrome c proceeds at a rate which is set by the exhaustion of the oxygen by the respiration of the cells.

As mentioned above, the measurement of oxygen concentration in the moist chamber is accomplished in two ways. For prolonged illuminations in which a uniform oxygen concentration is established throughout the layer of liquid covering the Chlorella, a platinum micro-electrode polarized at −0.6 V inserted in the side of the moist chamber is satisfactory. But in order to measure transient changes of oxygen concentration in the layer of Chlorella settled upon the lucite bottom of the moist chamber, a platinum spiral has been embedded in the lucite. This spiral was of sufficient diameter (~ 1 cm) to sample a large portion of the cell population. Also the projection of the electrode diameter above the base of the chamber in the horizontal plane (~ 0.1 mm) was sufficient to give some sampling in the vertical plane, since the thickness of the layer of settled cells was usually about 0.3 mm. A calomel electrode and salt bridge were used with this electrode. Thus, a sensitive and rapid response to the intracellular generation of oxygen is obtained as soon as the diffusion gradient has moved outside the cell surface. Changes of oxygen concentration of less than 10^{-7} M can readily be registered. The current from either of these electrodes is amplified and recorded by means of a chopper amplifier.

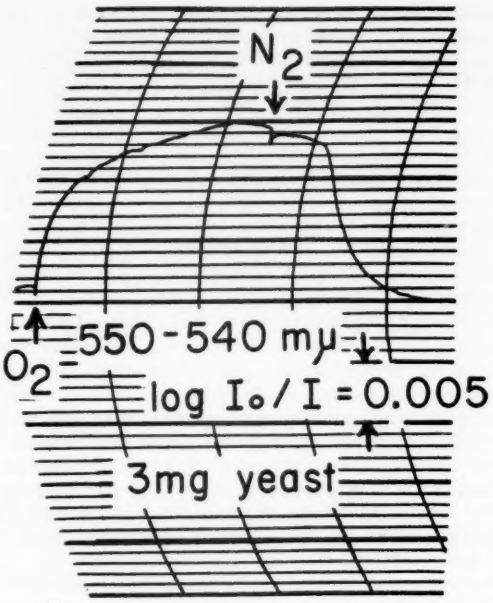

FIG. 2. An illustration of the response of cytochrome c of yeast cells to oxygenation and nitrogenation in the moist chamber of figure 1. The upward rise of the trace immediately following oxygenation is due to oxidation of reduced cytochrome c of yeast. There is a lag in the response following nitrogenation which corresponds to the time required to flush most of the oxygen out of the chamber. When the oxygen concentration has been reduced to a value corresponding to the affinity constant for cytochrome oxidase, reduction of cytochrome c occurs rapidly. The time interval between vertical lines is 60 sec. (632).

Values of light intensity used in these experiments are included only in order to allow others to approximate our experimental conditions. The values of lux given are those measured with a G.E. type DW-58 foot-candle meter and are the intensity values actually incident upon the lucite bottom of the moist chamber illuminated by the tungsten source via filters and mirrors as used. The light intensity was varied by neutral filters that were calibrated at 670 mμ.

The cell concentrations used here were comparable to those used by Duysens (17) who obtained absorbancy changes at 520 mμ about equal to those of figures 3 to 5. About 85 to 95 % of the light incident at 520 mμ is absorbed or scattered. These highly absorbing cell suspensions, which are necessary for obtaining adequate signal-to-noise ratio, lead to unhomogeneity of the red illumination, but this is minimized in this apparatus by the 400-mm² area of the moist chamber.

Materials

Chlorella pyrenoidosa was grown in a salt medium in daylight. This medium contained citrate as a carbon source, salts (Mg^{++}, NO_3^-, PO_4^{--}) and the usual trace elements, and was adjusted to pH 6.0. The culture flasks were one-liter " Shaker " type and only 200 ml of the medium was added. *Chlamydomonas reinhardii* (15) were grown in the light in salts, both with and without an acetate supplement. The algae were concentrated by centrifugation and were resuspended in the growth medium.

Results

Spectroscopic Effects of Illumination: In order to present the various spectroscopic effects of illumination in an understandable fashion, a number of separate experiments are presented under experimental conditions which are believed to represent separate effects clearly. In order to accomplish this, it has been necessary to correlate the spectroscopic effects, as measured by the double-beam spectrophotometer, and the extracellular oxygen concentration, as measured by the platinum electrode. Effects of illumination so far reported for Chlorella are mostly under aerobic conditions, particularly those described in the studies of Witt (7) and Strehler and Lynch (6). Thus, the first experiment to be considered is one which is intended to duplicate their conditions. In figure 3 B, the cells have been oxygenated by a succession of illuminations prior to the experiment reproduced here and the oxygen concentration at the beginning of the experiment corresponds roughly to 0.2 micromolar, a concentration which is adequate for the phenomenon being observed.

Illumination with 35 lux produces an abrupt downward deflection of the spectrophotometric trace corresponding to an increase of absorption of about 0.002 at 515 mμ measured with respect to 495 mμ. The oxygen trace indicates a transient rise and then a steady increase to 0.6 micromolar. Upon cessation of illumination, both traces return abruptly to the dark levels. The cell suspension remains aerobic. This small and rapid decrease of absorption caused by cessation of illumination is termed " the aerobic light effect " and is defined as a phase 2 transition.

If the cells are initially anaerobic due to flushing with 95 % N_2 – 5 % CO_2 gas mixture, their absorbancy at 515 mμ decreases from the level of figure 3 B to the level indicated in the left-hand edge of figure 3 A. Illumination with 35 lux now causes a much larger increase of absorbancy than in figure 3 B. There follows a slight decrease of absorbancy during the first 20 seconds following illumination. After brief transient, the extra-cellular oxygen rises to approximately 0.3 micromolar and upon cessation of illumination falls to zero in about half a minute. When the light is turned off, the spectroscopic trace shows a

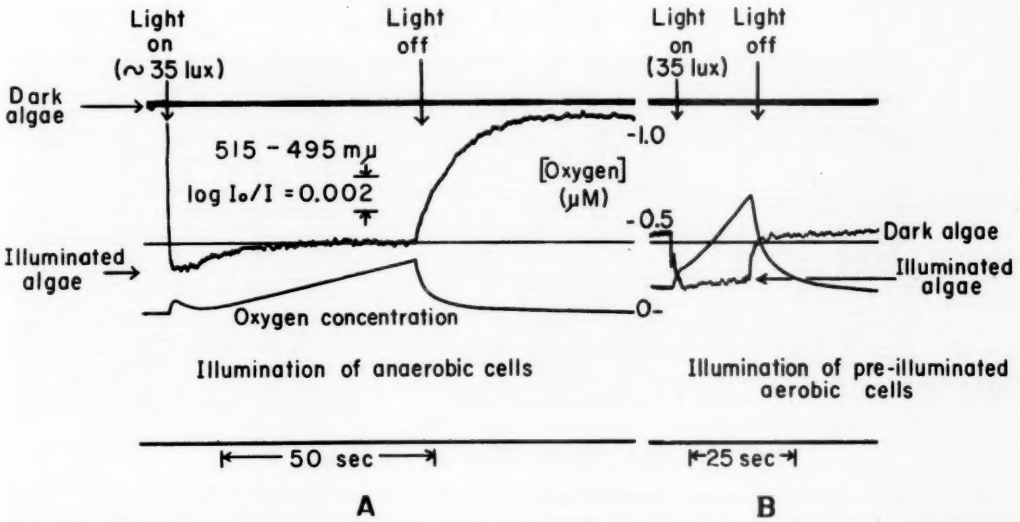

Fig. 3. Combined spectrophotometric and platinum electrode recordings of the effects of illumination upon Chlorella. This figure emphasizes the difference between illumination under anaerobic and aerobic conditions. At low oxygen concentrations in A, there is only the phase 3 transition upon cessation of illumination. Under highly aerobic conditions in B, only the rapid phase 2 transition is observed as described in text. (690).

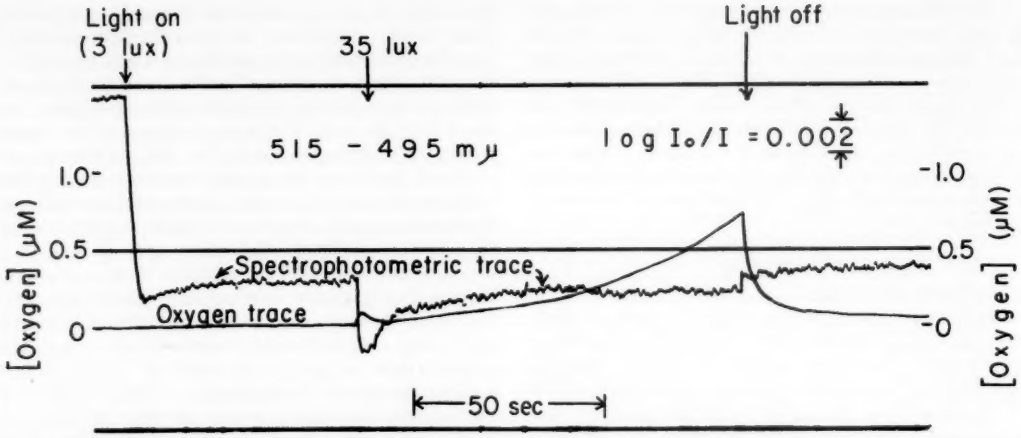

FIG. 4. Simultaneous platinum electrode and spectrophotometric measurement of the effects of illumination of a Chlorella suspension. The spiral platinum electrode is used to record the oxygen concentrations. This figure serves to distinguish between aerobic and anaerobic illumination of the algae. The phase 1 transition is represented by the slow decrease of the absorbancy at 515 mμ following illumination, and the phase 2 transition is the very abrupt decrease of absorbancy caused by cessation of illumination. The phase 3 transition is more clearly illustrated by figures 3 and 12. (690).

large rise indicating a decreased absorption at 515 mμ and at the end of about a minute has returned closely to the dark value. These spectroscopic effects are related to intracellular oxygenation of the Chlorella, due to photosynthetic activity. The decrease of absorption upon cessation of illumination is defined as a phase 3 transition.

The algae can be illuminated with such a low light intensity that intracellular spectroscopic effects are observed even though the extracellular oxygen concentrations cannot be recorded with the sensitivity available from the spiral electrode, and this is illustrated in figure 4. The algae are equilibrated with $N_2 - CO_2$ in the dark, and a light intensity of only 3 lux turned

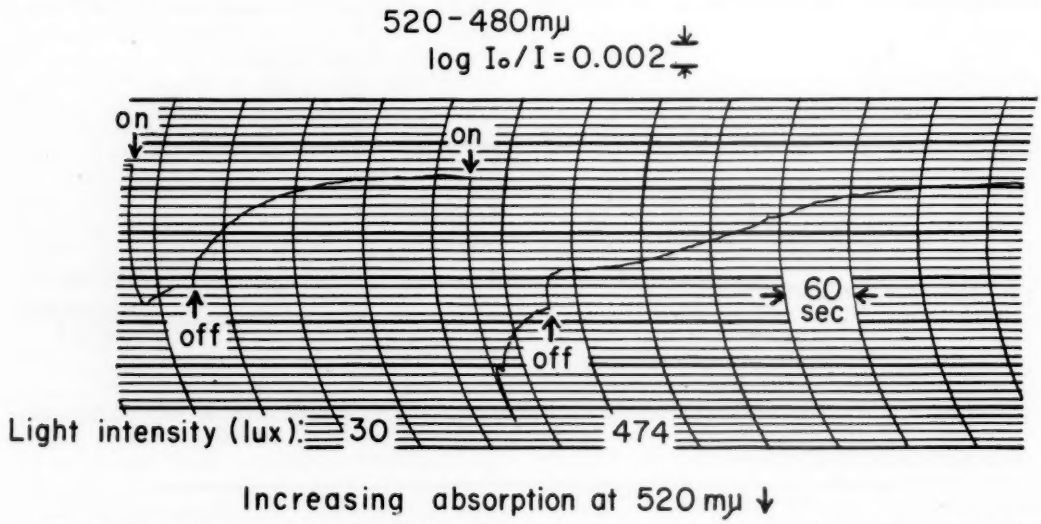

FIG. 5. Direct photographs of experimental records on the effects of red illumination upon Chlorella. On the left-hand side the light intensity is low and the absorbancy at 520 mμ relative to 480 mμ rapidly increases as indicated by the downward deflection of the trace. Following the steady state, the light is turned off and the trace returns to the base line. In the right-hand portion of the figure, an intensity of light is used that gives approximate saturation of the rate of oxygen production and absorbancy increases at 520 mμ are slightly larger than those at the lower light intensity. The reaction kinetics in this case involved the phase 1, 2 and 3 transitions. (623).

on. The characteristic increase of absorption at 515 mμ is observed although the oxygen trace remains horizontal. A ten-fold increase of light intensity causes an abrupt downward deflection of the trace, and in this case the deflection is definitely not constant, but falls back towards the level observed at the lower light intensity (this is a phase 1 transition). The oxygen trace, after a brief transient, shows a rise to a concentration of approximately 0.7 micromolar, at which point the light is turned off. The oxygen concentration under these conditions does not fall to zero but remains at a finite value (around 0.1 micromolar) until the end of the trace. The spectrophotometer shows only the "aerobic light effect" (phase 2 transition), and there is no phase 3 transition because enough extracellular oxygen remains to the end of the record to maintain the spectroscopic change.

Figures 3 and 4 should now afford a basis for understanding the complex cycle of spectroscopic changes that are recorded in figure 5. In this case the data are recorded on a slower time scale and at higher light intensities in order that the full cycle of events might be recorded. As in the previous figure, the cells are equilibrated with $N_2 - CO_2$. The left-hand portion of the record represents only a test of their response at a light intensity comparable to that used in figure 3, and the trace is in good agreement with the trace of figure 3 A. In the right-hand portion of figure 5 a much higher light intensity is used and the complex sequence of spectroscopic events that ensues is recorded. Upon illumination of the anaerobic cells the typical abrupt increase of absorption, measured in this case at 520 mμ with reference to 480 mμ, is observed, but the deflection is not stable and decreases to a steady value within a minute. This we define as the phase 1 transition and data below indicate that it may be related to the increase of the light requirement of the spectroscopic effect upon oxygenation of the cell suspension by photosynthesis (cf fig. 10). Upon cessation of illumination the small and rapid decrease of absorption at 520 mμ characteristic of the "aerobic light effect" (phase 2) is recorded. Since the cells were fully oxygenated by photosynthesis the trace is steady for about a minute while the excess oxygen is removed by dark respiration; then the absorption at 520 mμ slowly decreases as the extracellular oxygen falls to zero (phase 3 transition).

The absorbancy change that occurs upon illumination is not given a specific designation because it may consist of various combinations of the reverse of the phase 1, 2, and 3 transitions. For example, illumination of the anaerobic cells at low intensities (3 lux) as in figure 4 causes chiefly the phase 3 transition, while in figure 3 B only the phase 2 transition occurs.

With this introduction into the nature of the phenomena to be studied and the designation of the various spectroscopic changes, we shall now proceed to outline some control experiments and to indicate the spectra corresponding to one of the transitions caused by changes in illumination. We shall than return to the question of the relationship of the absorption changes and the oxygenation of the cells.

Effect of the Measuring Light: Since the response of the 515 mμ pigment to illumination under anaerobic conditions is extremely sensitive, there is a possibility that a portion of the spectroscopic change might already have occurred due to illumination with the measuring light and we have therefore repeated experiments similar to those in figure 3 at various values of the intensity of the measuring light and at a constant value of the exciting light. These data are plotted in figure 6 and it is seen that only at the highest value of the measuring light is there a measurable decrease of the spectroscopic effect. The conversion of the photocurrent at the photomultiplier anode to the light intensity in lux, as given in the figure legend, shows that 40 μa corresponds to only about 0.2 lux at 515 mμ.

The Effect of Light Intensity upon the Kinetics of the Absorbancy Change at 518 mμ. While Witt has made detailed studies of the kinetics of the absorbancy change caused by flash illumination of what are presumably the aerobic cells, no reports have yet appeared on the speed with which the absorbancy change occurs upon illuminating the anaerobic algae. A typical record of an experiment on the kinetics of this change is given by figure 7 which indicates the kinetics of the "on" and the "off" reactions for a given

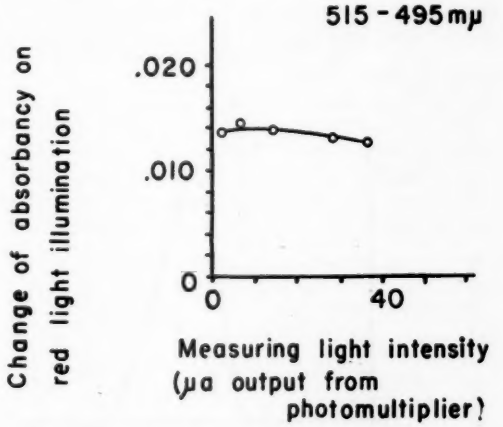

Fig. 6. The effect of the intensity of the measuring beam upon the change of absorbancy at 515–495 mμ at a constant intensity of red light illumination. The intensity of the measuring beam is given in terms of the photocell output current obtained through a Chlorella suspension. A developmental type K 1234 Dumont photomultiplier tube was used at 80 volts per stage, giving an approximate gain of 30,000. Thus the point 40 on the abscissa would correspond approximately to 1×10^{-9} amperes of primary photocurrent. At a sensitivity of roughly 2 amperes per lumen, the point 40 on the abscissa corresponds to 20×10^{-6} lumens. Averaged over the 1 cm^2 area of the moist chamber, 20×10^{-6} lumens corresponds to 0.2 lux. (62).

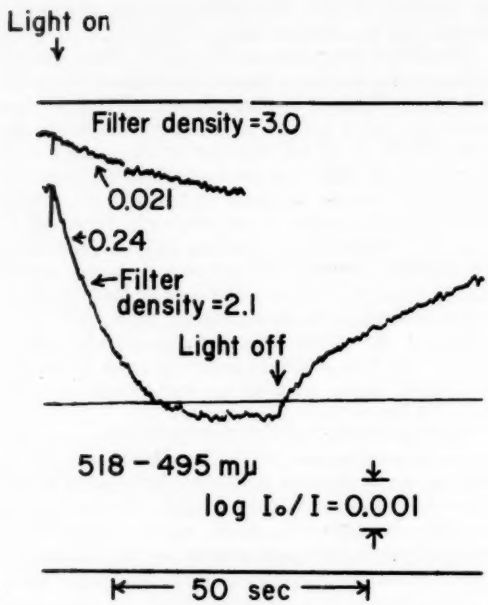

FIG. 7. Effect of a 10-fold variation of light intensity on the kinetics of the absorbancy increase at 515 mμ caused by illumination. The light intensity is varied by a neutral filter and the algae are initially in the anaerobic state. The slopes of the traces are in units of $10^3 \log \frac{I_o}{I} \Big/ \text{sec.}$ (625).

intensity of light and, in the upper portion, the kinetics of the "on" reaction for a ten-fold reduction of the exciting light intensity by means of a neutral filter. The ratio of the initial slopes of the kinetics of the two traces is 11:1, indicating reasonable proportionality between reaction kinetics and light intensity.

DIFFERENCE SPECTRA FOR THE EFFECT OF THE EXCITING LIGHT: In order to verify in detail the hypotheses based only on absorbancy changes measured at 515 mμ and 495 mμ, in the next two sections of this paper we compare the spectra for the illumination of the cells under anaerobic conditions with those obtained by oxygenation. Difference spectra corresponding to illumination of the anaerobic cells are necessary since it is uncertain as to whether cells were aerobic or anaerobic in previous works (3 to 8, 10), or in cases where the degree of aerobiosis was noted (7, 9), complete spectra have not been published. By taking measurements similar to those of figure 3 (left) (phase 3 transition) at a variety of wavelengths and by plotting the steady state deflection as a function of wavelength, we obtain difference spectra for the effect of low light intensities. This has been done for two types of algae.

Chlorella: In figure 8 A, we have plotted the absorbancy increases that occur upon illumination of Chlorella with red light. There is an absorption peak at 515 mμ, a plateau at 493 mμ, and a trough at 475 mμ. There is no distinctive effect in the region 550 to 555 mμ. The relationship of this spectrum to other work (3, 4, 6, 9, 10) is discussed below.

Chlamydomonas: The similar record for Chlamydomonas grown photosynthetically on a mineral medium shows a broader peak at approximately 518 mμ

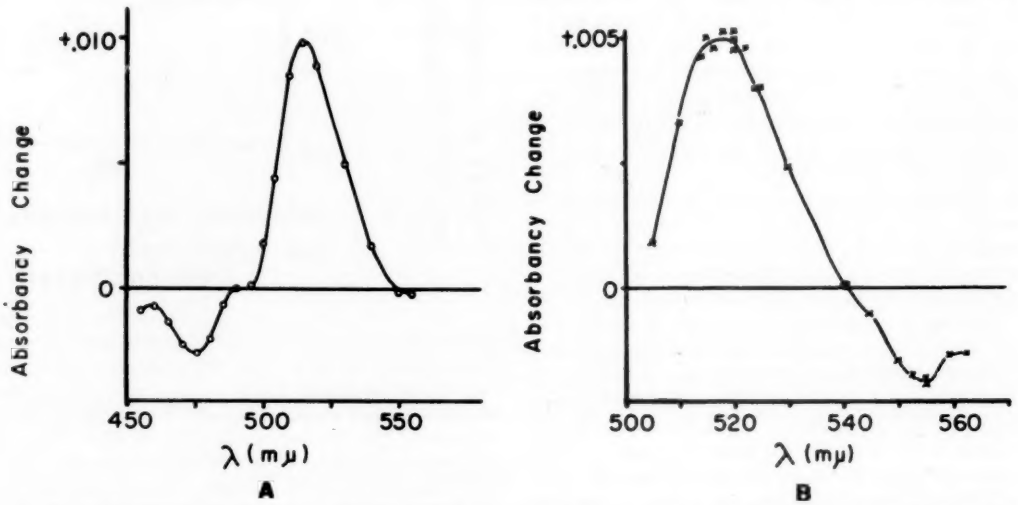

FIG. 8. Spectroscopic effects of illumination upon two types of green cell under anaerobic conditions (N_2 + 5 % CO_2). A. Chlorella. B. Chlamydomonas. The illumination used in these experiments was in general of lower intensity so that the kinetics resembled those of the left-hand portion of figure 5. The positive values of absorbancy change are plotted in an upward deflection, i.e., these are the absorption bands that appear upon illumination. Temperature in all experiments was 26° C. (668 and 666).

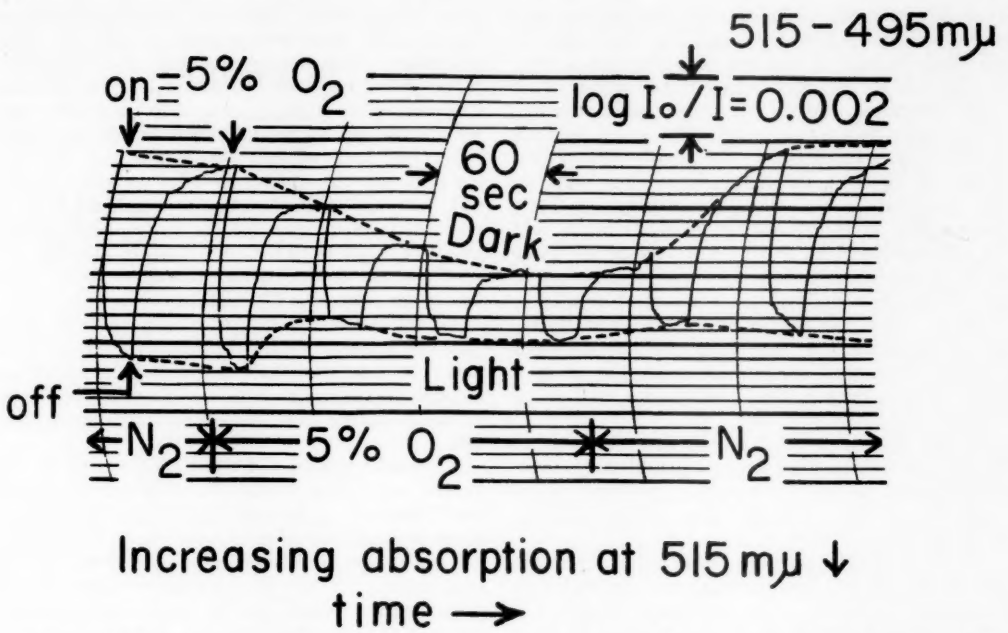

FIG. 9. The effect of oxygenation upon the absorbancy changes caused by low intensity illumination. The Chlorella suspension, initially in anaerobiosis, is repetitively illuminated throughout the experiment. After the first light-dark cycle, the gas mixture is changed to 5% oxygen and the attendant changes in responses to illumination are recorded. To serve as a control, 5% oxygen is replaced by nitrogen and the initial conditions are re-established. The conditions of the experiment are identical to those of the left-hand portion of figure 5. (626).

(fig 8 B), and in this case there is some absorption change in the region of 550 and 560 mμ. The trough between 550 and 555 mμ is so broad that it cannot be attributed solely to cytochrome f (see below).

EFFECT OF ADDED OXYGEN: We now return to the question of the relationship between oxygen concentration and spectroscopic effects. In figure 9 are recorded a number of cycles of spectroscopic changes caused by alternation of dark and light. The light intensity used is such that a phase 3 transition predominates (cf fig 3 A). After a trial period of light and darkness in the presence of nitrogen, the gas is changed to 5% oxygen, and after two minutes the amplitude of the light effect has diminished to about one-third and represents the smaller "aerobic light effect" (phase 2 transition). Upon readmission of nitrogen the larger phase 3 transition is established. The dashed curve is drawn on the figure to connect the extremes of the traces and to emphasize the change produced by oxygenation. The dashed line labeled "dark" indicates the increased absorbancy at 515 mμ, measured with respect to 495 mμ, caused by oxygen alone. The dashed trace labeled "light" indicates a change that may be related to the phase 1 transition —an increase of light requirement for the oxygenated cells, and hence a net decrease in the absorption of the illuminated cells in oxygen.

The phase 1 effect is explained by the graph of figure 10 in which the relationship between the intensity of illumination and the magnitude of the absorbancy change measured immediately after illumination under aerobic (open circles) and anaerobic (crosses) conditions. The latter is an unusual saturation curve, but is converted to a simple curve by subtracting the aerobic effect from the anaerobic one.

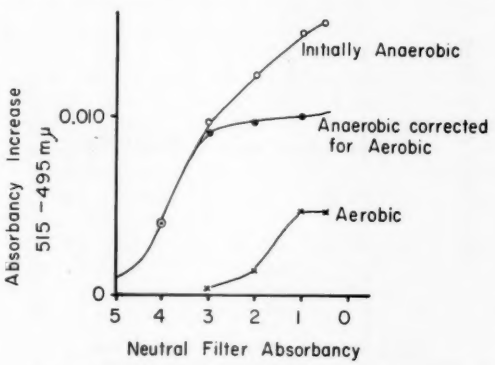

FIG. 10. Effect of illumination intensity upon the extent of the absorbancy changes at 515 mμ under aerobic and anaerobic conditions. The light intensities are varied by neutral filters. In order to compute the true anaerobic light effect the aerobic curve is subtracted from the anaerobic one. (701).

Thus we obtain the solid circles representing the basic anaerobic effect. It is now clear that approximately 100 times the light is required for half-maximal aerobic effects as for half-maximal anaerobic effects. Thus a cell suspension, initially anaerobic and illuminated with a strong light intensity, will show a slow decrease of absorption at 518 mμ as the photosynthesis proceeds. In figure 9, the lower dashed line indicates that oxygenation diminishes the response to light. The simplest hypothesis for the phase 1 effect is that the photosynthetically produced oxygen is the cause of the diminished response. However, illumination of the aerobic cells (fig. 4) gives an effect similar to phase 1. Thus the simple hypothesis must be extended to include the possibility that photosynthesis products other than oxygen can cause the phase 1 effect, or that more complex hypotheses need to be considered (6, 16).

So far as we have not shown that the phase 3 transition caused by illumination has a difference spectrum identical to that of the phase 3 transition caused by oxygenation by an external gas. This is taken up in the next two sections.

Oxidized Minus Reduced Difference Spectrum for Chlorella and Chlamydomonas: By repeating at various wavelengths the experiment of figure 9 without red illumination, we can record point by point the deflections obtained upon oxygenating the anaerobic algae in the dark. In figure 11 A, the oxidized minus reduced spectrum for a Chlorella suspension shows a peak very near to 515 mμ. Chlamydomonas grown in a medium containing acetate respire rapidly and become anaerobic soon enough after oxygenation to permit the use of a suspension of organisms in a 1-cm path cuvette. Since no red cross-illumination was used, no filters were needed to shield the photomultiplier. Thus the spectroscopic data of figure 11 B cover the range 490 to 648 mμ. This difference spectrum shows a sharp peak at 515 mμ, the typical shoulder at 530 mμ with a trough at 550 mμ, and no other very large changes out to 648 mμ. The 515-mμ peak and the 530-mμ shoulder are clearly identified with the characteristic light responses of the plants to oxygen. The 550 mμ trough is too broad to be attributable to cytochrome f alone, although it is possible that two or more cytochrome bands could fuse to give this broad trough.

A number of controls have been made to ensure that no artefact occurs during oxygenation in the dark. A more important one is that the absorbancy increase occurs just as rapidly and to the same extent if the measuring light is turned off during oxygenation.

Aerobic-Anaerobic Difference Spectrum Obtained by Illumination: Instead of oxygenating the Chlorella by a stream of gas as has been done in order to obtain the data of figure 11, it is possible to use the oxygen produced in photosynthesis for this oxygenation. In the first portion of figure 12, the measuring wavelength is at 507 mμ and in the second, 522 mμ, and 495 mμ is used as the reference wavelength in both cases. Illumination of the anaerobic algae for about 2 minutes at an intensity comparable to that of figure 5, right hand portion, causes a sharp increase of absorbancy at the measuring wavelengths, followed by the phase 1 decline of absorbancy attributed to the decrease of light sensitivity. On turning off the light, the abrupt phase 2 transition from il-

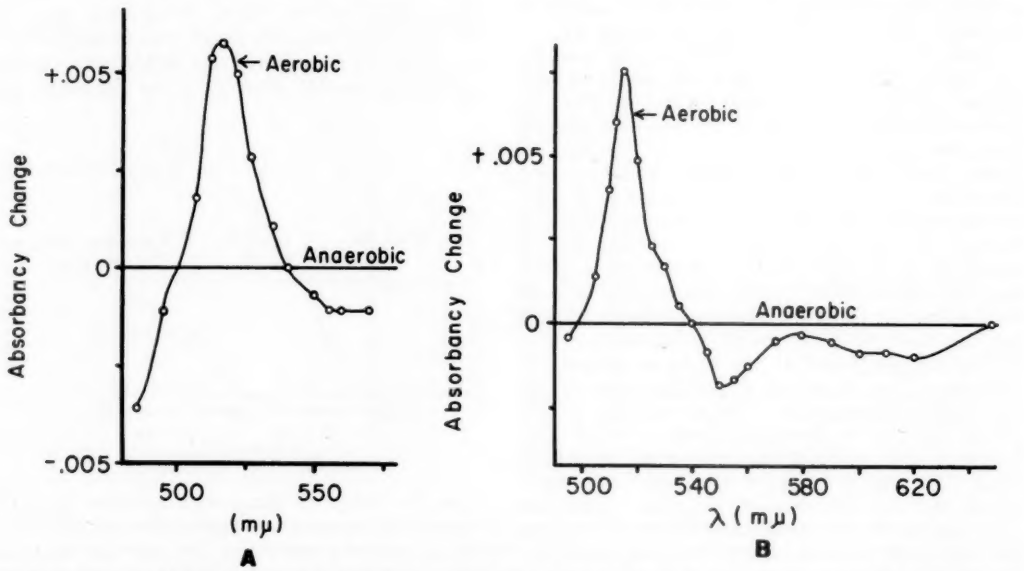

Fig. 11. Aerobic minus anaerobic difference spectra for Chlorella (A) and for acetate grown Chlamydomonas (B). The conditions of oxygenation were the same as those represented by figure 8 A. (666 and 668).

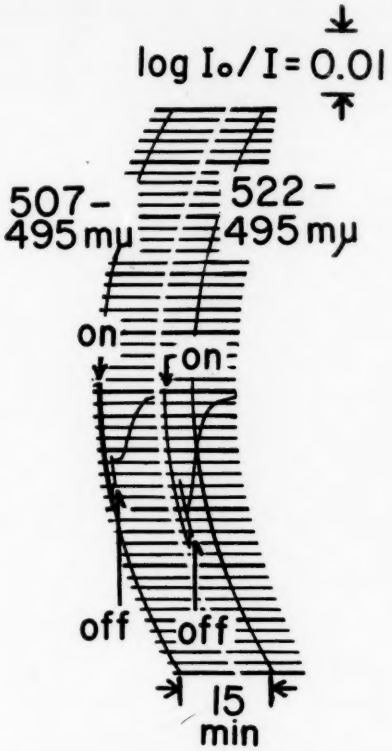

FIG. 12. Illustrating the use of the phase 3 transition to measure the oxidized minus reduced spectrum for Chlorella. Following the abrupt transition which occurs upon cessation of illumination (phase 2) the slow disappearance of the absorption band (phase 3 transition) can be followed at 507 and 522 mμ (cf fig 3). The measuring wavelength is shifted from 507 to 522 mμ at the dividing line in the figure. (707).

luminated to dark aerobic cells occurs. The cells are now in an aerobic dark state and the absorbancy changes in the next ten minutes represent phase 3 of figure 5 and are attributed to the disoxygenation of the cells. These two records show the effects of 522 mμ to exceed those at 507 mμ, and a repetition of these experiments at wavelengths in the region of 500 to 530 mμ gives a difference spectrum for the phase 3 transition that agrees with that of figures 8 A and 11 A to within the experimental error. Thus three methods for observation of the phase 3 effect give similar results: 1) oxygenation of the dark, anaerobic cells; 2) dim illumination of the anaerobic cells, and 3) the aerobic-anaerobic transition of cells following cessation of bright illumination.

DISCUSSION

The general effects of illumination of the green plant cell and purple bacteria present an interesting parallel that deserves discussion. Even though the bacteria do not form oxygen upon illumination, there is very clear evidence that an oxidant is produced, and, based upon recent information, this oxidant is powerful enough to oxidize not only the cytochromes of the respiratory chain but, in addition, to affect carotenoid pigments (Dr. Lucile Smith, (20)). It has also been found that the response to the cell depends upon the presence or absence of oxygen, since the spectroscopic effects observed in the anatrobic cells are greatly diminished and changed in nature by aerobiosis. In a companion paper (15), studies of a Chlamydomonas mutant are reported and there the illumination of the anaerobic cells oxidizes pyridine nucleotide and cytochrome b in a manner similar to, but less extensive than, that caused by oxygenation. These oxidations caused by illumination under anaerobic conditions are also diminished and changed in their nature under aerobic conditions. In studies of the normal green cell, we find a similarity of the basic phenomena. First, the magnitude of the spectroscopic effects produced by illumination is sensitive to added oxygen, as in the case of the two other systems: in Chlorella over 50 % of the absorbancy change at 515 mμ produced by illumination is caused by oxygenation in the dark. Second, oxygenation in the dark causes a spectroscopic effect in the region 475 to 540 mμ similar to (but smaller than) that caused by illumination with red light.

In the Chlamydomonas mutant, oxygenation in the dark causes spectroscopic changes similar to those recorded in other microorganisms and there is no difficulty in recognizing most of the pigments as members of the respiratory chain of the photosynthetic cells. Since illumination causes similar spectroscopic changes, identification of most of the pigments active in respiration and photosynthesis is not difficult.

In the mutant, the nature of the photochemical reaction can be elucidated: for example, the formation of an oxidant upon illumination is easily proved because the pigments involved are oxidation-reduction indicators (pyridine nucleotide, cytochrome b, etc.). But in the normal green cell, the pigment responding most clearly to red illumination is not recognizable as a member of the respiratory chain of any non-photosynthetic cell, nor are its properties as an oxidation-reduction indicator known. Thus, various criteria must be considered in order to establish the relative oxidation state of the substance responsible for the 518-mμ band:

1) Physical evidence for the formation of an intracellular oxidant upon illumination is obtained from observations of respiratory carriers in purple bacteria, a Chlamydomonas mutant, Porphyridium, and with less certainty, Chlorella (3).

2) No unequivocal evidence for the formation of the photochemical reductant has yet been obtained from spectroscopic studies of respiratory carriers of the uninhibited cells, purple bacteria, a Chlamydomonas mutant, or Chlorella. Although Duysens obtains suggestive evidence in recent fluorimetric studies of *Rhodospirillum rubrum* (18).

3) The 518-mμ absorption band persists after cessation of illumination when extracellular oxygen can be demonstrated (fig 4) and disappears approximately as the extracellular oxygen concentration falls to zero (fig 3 A).

4) An absorption band very similar to that obtained by low intensity illumination (phase 3 effect) is obtained by oxygenation of Chlorella and Chlamydomonas in the dark.

5) Studies of the respiratory carriers of various cells and mitochondria isolated therefrom have not shown any reductant to be produced upon oxygenation of the anaerobic cells.

On the basis of these data it is probable that the 518-mμ band identified in the phase 3 transition corresponds to a higher oxidation state. Since the disappearance of a band at 475 mμ is simultaneous with the appearance of the 518-mμ band, the 475-mμ is tentatively attributed to the reduced form of the pigment. The oxidant may be molecular oxygen or intermediates produced in water splitting (OH, etc.).

The phase 3 transition may differ from the "aerobic light effect" or phase 2 transition. First, about a 100 times more light is required for phase 2 than for phase 3. Second, extensive oxygenation of Chlorella does not suppress the phase 2 effect, as would be expected if the phase 2 effect were just a further oxygenation of the cells. Third, the difference spectra for high intensity illumination of the aerobic cells show bands that are clearly absent in the aerobic-anaerobic difference spectra (compare Kok's figure 1 (10) with our figure 11 and note the absence of the multiple bands in the region 560 to 640 mμ in our figure 11 B). It is very likely that additional components are involved in the phase 2 effect.

Witt (7) finds the phase 2 effect to disappear more rapidly on cessation of illumination in the presence of 2,6-dichlorophenol indophenol. While he interprets this result as evidence for a more reduced state in the phase 2 518 mμ-compound, the lack of an effect of ferricyanide or quinone requires an explanation. In fact the action of 2,6-dichlorophenol indophenol on energy-linked reactions is rather poorly understood (21, 22), and may not afford a definitive test of the oxidation state of the 518-mμ compound involved in the phase 2 effect. Thus theories which identify this substance with the reduced intermediate XH may be premature.

The dark form of the pigment from which the 518-mμ compound is derived in the phase 3 transition is unknown. It is very significant, however, that this absorption band does not appear upon illumination of a carotenoid-deficient Chlamydomonas mutant, while it shows clearly in the normal cells. A very similar relationship between a carotenoid-free mutant of purple bacteria and the normal cells has been demonstrated by Dr. Lucile Smith (personal communication). On this basis we can conclude that carotenoid is necessary for the 518-mμ band. It should be pointed out that the presence of carotenoid may not be a sufficient condition for the appearance of the 518-mμ absorption band because Duysens failed to demonstrate this band upon illumination of *Porphyridium cruentum* (4).

The function of the 518-mμ compound in photosynthesis has not been demonstrated, but there have been proposals that it may represent spectroscopic evidence of the primary process in photosynthesis (7, 10), and a proposal for its interaction with chlorophyll has recently been made (16). In addition, recent data show chlorophyll-carotenoid interaction in vitro (23). The phase 3 transition may not be involved in a primary process since it can be caused by a dark reaction (oxygenation). Neither the phase 2 or 3 transition is observed in the Chlamydomonas mutant in which active photosynthesis occurs, and hence the 518-mμ compound may not be required for photosynthesis. A possible function for this compound in the protection of the cell from excess oxidizing equivalents is discussed elsewhere (15).

There now exist a number of difference spectra for the illumination of Chlorella and significant differences are beginning to be established. The two spectra which differ markedly from those obtained by Duysens (4), Spruit (9) and this work are those obtained by Strehler and Lynch (6) and by Kok (10). The last two studies have an important feature in common: they were both based on measurements of the amplitude of the dark reaction at arbitrary times after bright illumination. Thus a wide range of wavelengths was covered. A disadvantage is that the complete reaction kinetics in light and dark could not be recorded. Strehler and Lynch used a flow system and Kok used a rotating shutter; both precluded obtaining data during illumination. Thus the relative sizes of the peaks in their spectra depend upon the assumption that the nature of the reaction kinetics does not change with wavelength. For example, Strehler and Lynch recorded a portion of the dark reaction kinetics that they termed "negative overshoot" which causes their results to be inverted (cf reference 5). In addition, Strehler and Lynch find that the "negative overshoot" has different kinetics at 525 and 648 mμ (6). In Kok's method, a decrease in the rate of the dark reaction at different wavelengths would cause an increase in the reading given by his instrument and thereby over-emphasize the relative magnitude of the slower dark reactions. Technically this is because Kok's first electronic switch cannot open "immediately after the flash"; a certain delay must surely be present, during which a variable decay of the spectroscopic effect may occur. Thus, although the methods cover a wide range of wavelengths at high sensitivity, the relative sizes of the peaks should be controlled by an apparatus of the type used by Duysens or by the double-beam instrument of the type described here.

The "negative overshoot" phenomenon (6) does not appear in these experiments in Chlorella, a result that is apparently in agreement with those obtained by others (7, 9, 10, 17). Thus further studies of the

conditions under which Strehler and Lynch obtained their results are desirable.

Spruit (9) finds that anaerobiosis causes a marked decrease of the 518-mμ band, a result contrary to Duysens' (8) and to the more detailed results of this paper. Since Spruit has not yet published a spectrum for the effect he has observed, it is difficult to compare his result with ours. On a technical basis it should be pointed out that the compensating beam method he used is very sensitive to non-specific light absorption effects that might have been altered by the establishment of anaerobiosis in Spruit's studies. This is because the "compensating beam" does not pass through the sample.

Lastly our data show no change in either the aerobic-anaerobic or the phase 3 difference spectra that can surely be attributed to an oxidation of cytochrome f upon illumination of the anaerobic cells. The absorption changes in this region of the spectrum are too broad to be attributed with any assurance to cytochrome peaks. Nevertheless the evidence from other workers for the participation of a cytochrome in the phase 2 effect is convincing (4). Whether this cytochrome is of type f or c is not clear (for a discussion, see (15)).

In summary, the spectroscopic changes that are caused by illumination of Chlorella are complex and depend upon the metabolic state of the cell which is very sensitive to illumination. On the technical side, these effects are large enough so that they can be measured by a number of spectrophotometric methods, each capable of giving somewhat different results. It is a matter of great importance to control both the biological and the physical aspects of studies of this interesting phenomenon so that meaningful and consistent data can be obtained, and that concordant interpretations of the nature of these effects can be achieved.

Summary

The spectrum corresponding to the differences of absorption between aerobic and anaerobic suspensions of Chlorella and Chlamydomonas have been recorded with a double beam bichromatic instrument. Instead of a cytochrome spectrum we find that oxygenation causes a major absorption band to appear at 518 mμ and a small band to disappear in the region of 550 mμ; the latter band is considered to be too broad to be attributed with certainty to cytochrome oxidation. These absorption bands are very similar to those which appear upon dim illumination of anaerobic cells (phase 3 transition). It is concluded that the absorption changes caused by dim illumination are associated with oxygenation of the cells. The spectroscopic effects caused by oxygenation and by low intensity illumination are found to differ in significant details from those caused by high illumination of the aerobic cells (phase 2 transition) where considerably higher light intensities are required for saturation and in which additional absorption bands are observed by other workers. A comparison of the properties of normal cells showing light- and oxygen-induced spectroscopic changes at 518 mμ with those observed in a carotenoid-deficient mutant which does not show such a change suggest that carotenoid is required for the 518-mμ absorption band (cf 6). Similar considerations suggest that the 518-mμ absorption band observed on oxygenation or low intensity illumination may not be required for photosynthesis. The identification of the phase 2 transition with the primary process of photosynthesis, as postulated by other workers, requires further study.

These studies emphasize the need for proper choice of the physical method and adequate control of the metabolic state of the photosynthetic and respiratory systems of the cell in order that concordant interpretations of the spectroscopic data can be made.

Thanks are due to Dr. Ruth Sager for the Chlamydomonas cultures.

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OXYGEN AND LIGHT INDUCED OXIDATIONS OF CYTOCHROME, FLAVOPROTEIN, AND PYRIDINE NUCLEOTIDE IN A CHLAMYDOMONAS MUTANT[1,2]

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In green cells direct spectroscopic studies of the respiratory pigments involved in oxidation-reduction reactions are difficult because their absorption bands are obscured by those of the photosynthetic pigments. Visual spectroscopy has been used by Hill and by Davenport (1, 2) on etiolated leaves and on cytochromes extracted from them and from green algae, but no studies comparable to the classic ones of Keilin on non-photosynthetic systems have yet been carried out. Spectra representing absorbancy changes caused by illumination have been reported by Duysens (3, 4, 5) and by Chance and Strehler (6) for Chlorella. Spectra representing changes immediately following cessation of illumination have been reported by Strehler and Lynch (7), and long persistent changes following a previous illumination are reported by Lundegårdh (8). Witt has studied in detail the kinetics of spectroscopic changes at 520 and 480 mμ (9). Inconsistencies in the results so far obtained indicate the necessity for a comprehensive study of spectroscopic effects in the intact cell. Duysens, who first found increased absorbancy at 515 mμ upon illumination of Chlorella, later found the effect to be lacking in Porphyridium. He further reported that cytochrome f was oxidized upon illumination of Chlorella on the basis of an absorbancy decrease at 420 mμ, an observation which was later verified in Porphyridium, but, in that case, he found, corresponding to the Soret band at 420 mμ, an *a* band at 555 mμ. This *a* band agrees with that of purified cytochrome f, but the Soret band differs by 4 mμ, a discrepancy beyond the experimental error.[3] This inconsistency in the identification of the cytochrome involved also applies to the question of whether pyridine nucleotide has been observed to be affected by illumination (4). Broad and non-specific increases of absorption in the ultra-violet region were observed for Porphyridium (4) and were attributed to increased reduction of pyridine nucleotide even though no 340 mμ peak was observed. Lundegårdh, using slower methods than any of the other authors, finds that oxidation of cytochrome f following illumination must have persisted (according to our estimates) for at

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[3] A close reading of Davenport and Hill's graph (19) gives 424 mμ as the correct wavelength and this value is used by Duysens (4).

least 30 seconds.[4] The cause of some of these discrepancies may be the failure to recognize aerobiosis or anaerobiosis as a factor affecting the nature and extent of the spectroscopic effects caused by illumination, even though this had already been shown to be of importance in the studies of *Rhodospirillum rubrum* (10). The possible importance of anaerobiosis was suggested by Duysens' recommendation that the cells be allowed to stand in the dark for "half a day" in order to enhance the spectroscopic effect of illumination and by his later note regarding the enhancement of the light-induced increase of absorption at 515 mμ by anaerobiosis (5).

The cytochromes that might participate in respiratory or in photosynthetic electron transfer in green and etiolated plants have been studied intensively by Hill and his collaborators (1, 2, 11). Cytochromes c, b_3, f, and b_6 have been isolated and interest has centered about the possibility of cytochromes f and b_6 participating in the photosynthetic process. The relative values of their oxidation-reduction potentials led Hill to speculate that b_6 would be completely reduced even in the presence of oxygen and that cytochrome f is oxidized in the illuminated leaf. An absorption band has been observed in the leaves of the golden varieties of certain plants in the position appropriate to reduced cytochrome b_6, although this absorption band was not observed in the chloroplast preparation unless dithionite was added.

It is apparent that spectroscopic data on the respiratory chain of the green cell together with the aerobic and anaerobic effects of illumination are necessary in order to give a comprehensive picture of the interaction of the respiratory and photosynthetic processes. While the oxidized minus reduced spectrum of Chlorella shows chiefly the 515 mμ pigment and very little indication of cytochrome, the studies on a pale green mutant of Chlamydomonas having low concentrations of carotenoids and chlorophyll (26) show clearly difference spectra of cytochrome, flavoprotein, and pyridine nucleotide components that are involved to varying extents in respiratory and photosynthetic reactions. The response of these components to aerobiosis and to anaerobiosis suggests their participation in the respiratory chain and their response to illumination under aerobic and anaerobic conditions may identify those components involved in photosynthesis. The importance of oxidation reactions caused by illumination is emphasized and the relative speeds of response of the components to illumination gives preliminary indication of the sequence of their reactions.

[4] Lundegårdh's recorder (8, 22) is a Leeds and Northrop device that plots one point every 2.2 seconds, and would require at least 30 seconds to record, at intervals of about 2 mμ, a spectrum from 540 to 570 mμ. The scanning mechanism described (22) requires an interval of 4 seconds between readings or a total interval of a minute. A personal communication from Lundegårdh verifies that by "a few seconds" (8) he meant 10 to 20 seconds. In this time interval after cessation of illumination, our rapid recordings show that the oxidation of cytochrome f directly caused by illumination would have fallen to a small fraction of its initial value, to less than 10%. One could propose that Lundegårdh recorded this last 10% of the light reaction, but his figure 3 (8) shows that ascorbate reduction gives "completely reduced" bands of cytochromes c and f and that these absorbancy changes are very nearly identical in magnitude to those recorded upon illumination. Thus, the hypothesis that Lundegårdh observed the same phenomenon as Duysens and we, appears to be untenable and the hypothesis proposed here that he observed the persistence of oxygenation of a previously anaerobic Chlorella suspension fits nicely with his own data. This confusion was caused by lack of adequate controls on oxygen concentration during experimentation with living cells.

Methods

Measurements were carried out with a double-beam differential spectrophotometer (12) fitted with an especially designed moist chamber described in the preceding paper (6). Since a wide range of wavelengths was covered in these experiments, a Wratten 39 and a Corning 978½ filter was used, the latter serving to eliminate the infra-red transmission of the former. This filter combination permitted measurements down to 320 mμ with an acceptable signal-to-noise ratio, and an excellent signal-to-noise ratio from 340 to 480 mμ. The filter combination also minimized stray light effects in the grating monochromators. For observations in the ultra-violet region, the tungsten source was operated at a considerable over-voltage in order to give a better emissivity. For studies of the visible region of the spectrum, the most satisfactory filter combination was Corning 430 plus 978½. For red illumination, Corning 2030 filter provided adequate intensity for saturating effects which were readily controlled by the insertion of neutral filters.

As in the previous paper (6), the oxygen concentrations were altered by passing either nitrogen or oxygen mixtures over the algal suspension. Although 5% CO_2 was present in the nitrogen and oxygen, it was apparently not necessary for the effects upon cytochromes b and f. In one test, pure nitrogen was passed over the cells for two hours with no evidence of CO_2 lack, but it is possible that a longer interval might have produced a demonstrable effect.

The moist chamber was filled as described in the previous paper (6). The mutant cells settled to the bottom of the chamber in a uniform film of several tenths of a millimeter thickness. The wild type formed an irregular film. The respiration of the suspension was measured by the two types of platinum electrodes described previously (6). The Chlamydomonas cells respired so rapidly that records of their oxygenation and disoxygenation resemble those obtained with yeast (cf fig 2 of (6)). Prolonged oxygenation of the suspension was required to oxygenate completely the respiratory system and to eliminate the "anaerobic light effects."

Carbon dioxide utilization by the cells was measured in terms of the decrease of acidity. A suitable apparatus for such changes was described previously for simultaneous measurements of respiration and fer-

mentation in suspensions of yeast cells (12) and Spruit and Kok have applied similar methods to O_2 and CO_2 exchange in Chlorella (27, see also 13). Suitable controls (fig 2) provided direct calibration of the sensitivity of the bicarbonate-buffer cell suspension system. The effects of heating the solution by the lamp as well as loss of CO_2 to the atmosphere were found to be negligible compared to the changes caused by photosynthesis of the algae. The effect of electrode polarization upon the potential of the glass electrode was controlled by periodically removing such polarization voltage and noting the effect upon the electrode potential. The same 100-watt tungsten projection lamp was used to illuminate the moist chamber as was used to illuminate the cuvette in these studies.

Materials

The pale green mutant (strain no. 95) was obtained in 1951 after ultra-violet irradiation of the green alga *Chlamydomonas reinhardii* and has been maintained subsequently on an acetate medium in the dark. This strain has never back-mutated to the normal green. For these experiments cultures were grown in the dark on the acetate medium (25) and harvested at the end of the growth cycle. The cells were centrifuged and resuspended in their own medium or in 0.01 M $NaHCO_3$. The moist chamber requires 1.5 ml of a cell suspension the density of which was adjusted to approximately 20 micromolar chlorophyll.

For comparison of photosynthetic activity, the normal green algae were grown in the presence of acetate in the light and in the dark, and the cells were prepared as described above and suspended in a bicarbonate buffer. The normal green strain used was the one from which the pale green mutant had been obtained.

Results

Extractable Pigments: The carotenoid and chlorophyll content of the mutant is indicated by the graph of figure 1, which is a recording of the absolute spectrum of an ether solution of pigments extracted by treatment of the cells with 80 % acetone. It is seen that there are major absorption peaks near 660 and 430 mμ. At the sensitivity used in recording these peaks, no absorption bands can be detected that might be attributed to carotenoid, but if the scale is multiplied ten times, a small shoulder is seen on the 430 mμ peak at about 480 mμ which is probably due to carotenoid. In a study of pigment content of the pale green mutant (26), it was found to contain only 1/500 the total carotenoid of normal green dark-grown cells, and about 1/10 the chlorophyll. The only carotenoids detected in extracts of a one gram (dry wt) sample of pale green mutant cells were alpha and beta carotene. In the normal green cells, these two pigments account for about 65 % of the total carotenoids. The mutant has a chlorophyll to carotenoid ratio of 180 : 1 (mole basis) while the normal cells have a ratio of 15 : 1 when grown in the light and only 4 : 1 when grown in the dark. On the basis of beta carotene only, these ratios are respectively 240 : 1, 24 : 1, and 7 : 1. Thus the mutant has less than 1/30 the β-carotene relative to chlorophyll of the normal green dark-grown cells.

An approximate value of the cytochrome content relative to that of chlorophyll is provided by a measurement of the ratio of the absorbancy change at 430 and 405 mμ caused by the transition from aerobiosis to anaerobiosis to the chlorophyll absorption at 430 mμ in 80 % acetone. The ratio is 1:12 and has roughly the same value when converted to a mole basis. This value indicates the relatively favorable

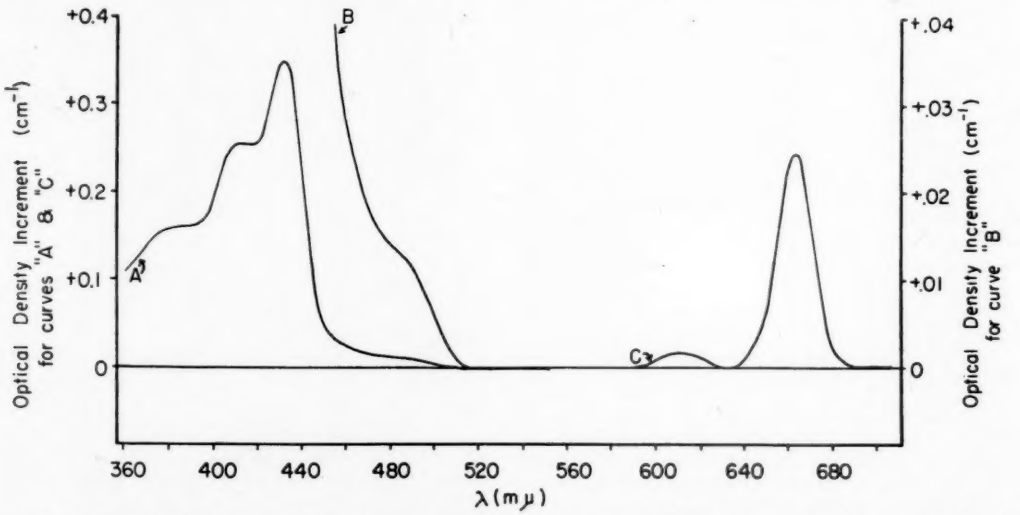

Fig. 1. The absolute spectrum of an extract of the total amount of Chlamydomonas mutant used to obtain the spectrum of figure 10. Curves A and C are recorded at the same sensitivity, and B is amplified 10-fold. (674 d).

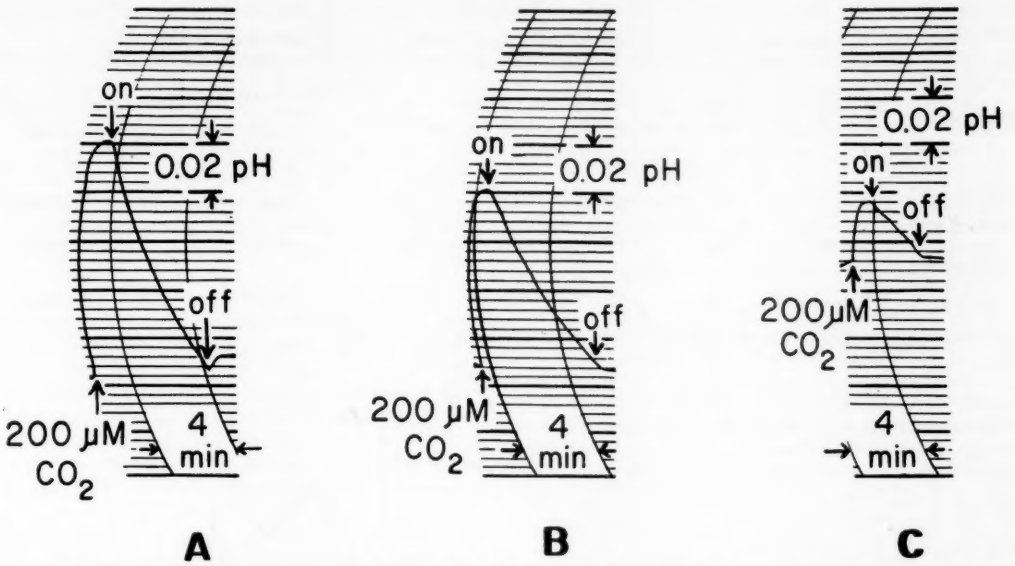

FIG. 2. A quantitative determination of the rate of CO_2 uptake by 3 different types of Chlamydomonas. The CO_2 uptake is measured by the pH change in a 0.01 M $NaHCO_3$ solution in a 2.8-ml cuvette. The electrode sensitivities and time scales are included in the diagram as well as the initial values of pH. The upward deflection of each of the three traces marks a calibration by the addition of 200 micromoles CO_2. The interval of illumination by 110 ft-c tungsten light is also indicated. A. Normal cells are grown in the light in acetate medium. B. Normal cells are grown in the dark in acetate medium. C. The pale green mutant is grown in the dark in acetate medium. (710 f).

conditions provided by the pale green mutant for observation of the cytochromes.

PHOTOSYNTHETIC ACTIVITY, *Carbon Dioxide Fixation:* The carbon dioxide fixation by three types of Chlamydomonas is illustrated by figure 2. The carbon dioxide uptake of the illuminated cells is measured in terms of the change of pH of 0.01 M $NaHCO_3$ solution by the glass electrode inserted into an open cuvette. As contrasted with the recent method of Rosenberg (13) whereby the conversion from pH change of carbon dioxide concentration was calculated for a solution free of interfering substances, we calibrate directly by the addition of a known volume of a saturated solution of carbon dioxide as do Spruit and Kok (27). Thus, the three records start with the addition of the calibrating solution to the dark cell suspension. This causes a decrease of pH (an upward deflection of the traces) and the amplitude of the deflection is indicative of the buffering capacity of the various suspensions. In the case of both the dark-grown green cells (B) and the pale green mutant (C), some acetate was present from the growth medium. The differences of these calibrations illustrate the importance of using this method (cf Rosenberg 13, table I).

As soon as the pH change due to addition of the calibrating solution is completed, the light is turned on and CO_2 uptake begins, prior illuminations having reduced the induction effects to a negligible value. The illumination is maintained until an amount of CO_2 equal to the calibration solution has been taken up. Then the light is turned off, and as shown by the traces, the CO_2 uptake comes to a halt. The rate of CO_2 uptake is simply the quotient of the concentration of CO_2 added in the calibrating solution (200 micromolar) and the number of seconds required to utilize that amount of CO_2. After the activities have been determined, the samples are treated with 80 % acetone, centrifuged, and the chlorophyll estimated in the Beckman spectrophotometer at 660 $m\mu$. These values were divided by the molecular extinction coefficient for chlorophyll a (82 $cm^{-1} \times mM^{-1}$ (14)). In the normal cells, chlorophyll b is also present and the concentration found for chlorophyll a is multiplied by 1.7 to give the total concentration. In the mutant cells, the correction factor is only 1.1. The photosynthetic activity is given in terms of chlorophyll turnover—μM CO_2 sec^{-1} $chlorophyll^{-1}$. The data are summarized in table I, and it is seen that the turnover number of the chlorophyll increases from the normal, to the dark-grown green cells, to the pale green mutant cells. These turnover numbers for chlorophyll are the reciprocal of the "assimilation time" and an inspection of Rabinowitch's table 28 V (15) shows that the values of table I agree very closely with those obtained for a wide range of leaves and algae; the best value that he gives for Chlorella is 0.03 and agrees very well with the values obtained for the light- and dark-grown Chlamydomonas.

Since the ratios of chlorophyll to β carotene con-

TABLE I

COMPARISON OF CO_2 FIXING ACTIVITIES OF THREE FORMS OF CHLAMYDOMONAS

DESCRIPTION	CONDITIONS OF MEASUREMENT	INITIAL pH	$\mu M\ CO_2$/SEC IN LIGHT	D_{680} (CM^{-1})	ϵ_{680} ($CM^{-1}MM^{-1}$)	$CO_2 \times$ CHLOROPHYLL^{-1} $\times$ SEC^{-1}	RATIO OF CHLOROPHYLL TO CAROTENE B	$CO_2 \times$ CAROTENE B^{-1} $\times$ SEC^{-1}
Light grown normal	Aerobic	7.7	−0.5	1.9	82	−0.02	24	−0.48
Dark grown normal	Aerobic	7.6	−0.5	1.4	82	−0.03	7	−0.21
Dark grown pale green mutant	"Anaerobic"	7.4	−1.0	1.1	82	−0.10	240	−24.00

Cells resuspended in 0.01 M $NaHCO_3$. White light, ~ 1000 ft-c. (710 f).

centration in the three types of cells are available, it is possible to calculate the turnover number of β carotene, assuming that it is participating in photosynthesis. It is seen that the value for the pale green mutant would have to be 100 times greater than that for the dark-grown normal cells. The significance of this result is discussed below.

Oxygen Evolution: Due to the relatively high respiratory activity of the pale green mutant under the conditions of figure 2, these cells were anaerobic at the time of illumination and the increase of oxygen concentration in the medium was too small to be measured by the platinum electrode. By passing a mixture of 5 % oxygen and 95 % nitrogen over the surface of the washed cells in the moist chamber, the oxygen concentration in the medium was raised to the point where the spiral platinum electrode could be used to demonstrate evolution of oxygen upon illumination. A typical record of both platinum electrode and spectrophotometer traces is shown in figure 3. The record begins at the left-hand edge with the cell suspension in the dark and both traces horizontal. Upon illumination, there is a disturbance of the spectrophotometric trace and then an abrupt upward deflection which corresponds to a decrease of light absorption at 430 mμ measured with respect to 450 mμ. This corresponds to an oxidation of cytochrome of

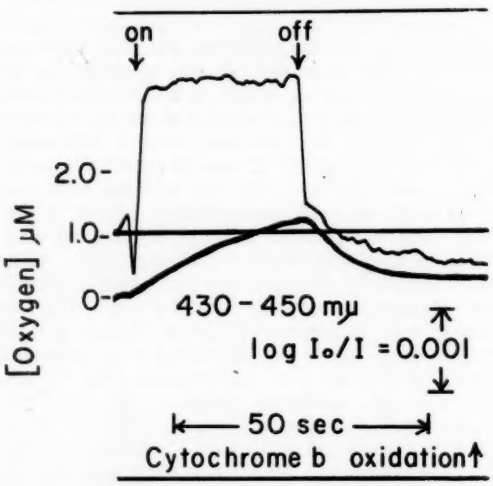

FIG. 3. Evidence for oxygen evolution caused by illumination of the pale green mutant. The cells are contained in the moist chamber described in the text and the oxygen concentration (lower trace) is measured by a spiral platinum electrode located in the lucite bottom of the chamber and polarized at −0.6 volts. The calibration for the oxygen concentration is given in the diagram and the upper trace represents a spectroscopic recording of the simultaneous absorbancy change by the double beam spectrophotometer. (702 b).

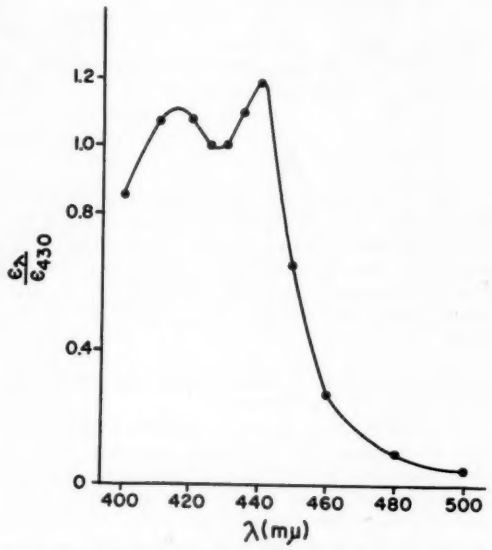

FIG. 4. The relative effectiveness of wavelengths of light appropriate to chlorophyll and carotenoid in photosynthetic oxygen evolution in the pale green mutant. The method used is that of Castor and Chance (17). (Experiments carried out in collaboration with Mr. Selwyn Ramsay.) (754).

type b. Shortly after illumination, the platinum electrode trace rises indicating an increased oxygen concentration due to photosynthesis in mutant cells. Upon turning off the light, an abrupt reduction of cytochrome b occurs and is followed by a fall of oxygen concentration due to dark respiration. The increase of oxygen concentration in the light and its utilization in the dark, together with the correlation of this change with the oxidation and reduction of intracellular cytochrome b, give further support to the idea that the mutant cells have an intact photosynthetic system.

Relative Effectiveness of Chlorophyll and Carotenoid Pigments in Photosynthesis: In order to determine the extent to which carotene pigments of the pale green mutant contribute to the photosynthetic activity, we have used the apparatus described by Castor and Chance (17) for measurement of the action spectrum for photosynthesis in the region of absorption maxima for chlorophyll and carotenoid pigments (fig 4). The wavelength of the peak of this spectrum indicates the participation of chlorophyll in oxygen evolution by the mutant cells. The sharp decrease of the effectiveness of longer wavelength of light indicates that carotenoid is not important in photosynthesis in the mutant cells.

Spectroscopic Effects of Aerobiosis and Anaerobiosis: The spectrum representing the absorbancy changes that occur in the transition from the steady state aerobic condition to the steady state anaerobic condition of the algal suspension is given in figure 5. It should be noted that the scale is broken at 500 mμ and the absorption bands in the visible region are plotted at twice the scale of those in the Soret and ultra-violet regions.

A number of components similar to those of the mammalian respiratory chain are immediately recognizable from the data.[5] Reduced pyridine nucleotide shows a symmetrical absorption band centered at about 345 mμ. Flavoprotein shows a distinctive trough at 470 mμ; the trough is caused by the disappearance of the oxidized form of flavoprotein. Cytochrome b shows a distinctive a band at 563 mμ and its corresponding Soret band at 430 to 431 mμ. An a-band attributable to cytochrome of type c shows clearly at 551 to 552 mμ. The Soret band of cytochrome c does not show clearly because it is relatively less distinctive. The cells show very unusual

[5] In order to clarify what may seem to be arbitrary relationships between the positions of the absorption bands of components of the electron transfer systems and the nature of the enzyme systems involved, we have made the following list:

a. Isolated diphospho- and triphospho-pyridine nucleotide have the absorption peaks of their reduced forms at 340 mμ and our spectra present the sum of the reduced forms of such pigments.
b. 430 and 563 mμ—these absorption peaks are attributed to cytochrome of type b without an attempt at this point to distinguish among cytochrome b of the respiratory chain, cytochrome b_3 and cytochrome b_6.
c. 424 and 555 mμ—absorption bands having their peaks at these wavelengths will arbitrarily be attributed to cytochrome f, although this may not be an unique or a final designation.
d. 420 and 550 mμ—pigments having their absorption bands at these wavelengths are identified with cytochrome c of the respiratory chain.
e. 450 to 470 mμ is the region where the oxidized form of flavoprotein absorbs and the pigment studied here is assumed to be that involved not only in the respiratory chain but also in the photosynthetic processes.

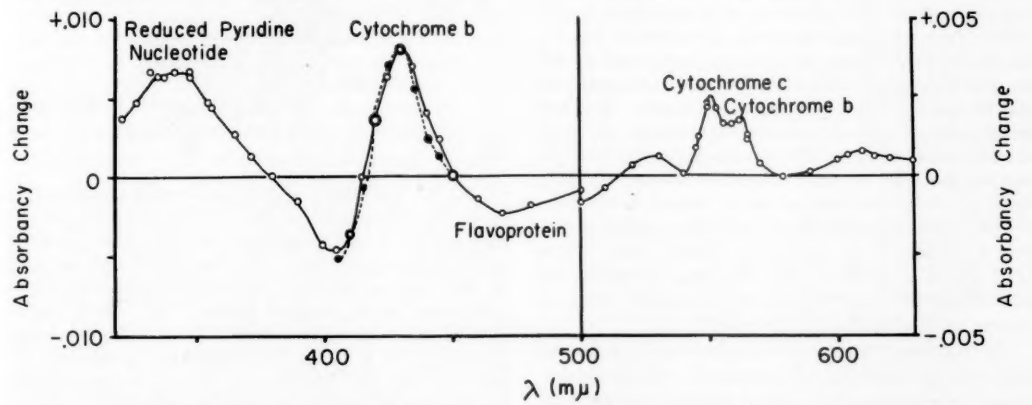

Fig. 5. The reduced minus oxidized difference spectrum for the respiratory components for the Chlamydomonas mutant. The open circles represent data taken by alternately flushing oxygen and nitrogen over the cell suspension with consequent oxidation and reduction of the pigments (for an example of a recording at a particular pair of wavelengths, see fig 6). A preliminary identification of the cytochrome components is included in the figure (cf footnote 3). Note that the magnification of the scale has been doubled in the visible region of the spectrum. The effects of alternately flushing CO and O_2 are illustrated by the closed circles in the region of the Soret band. (672).

TABLE II

RELATIVE ABSORBANCIES AND CONCENTRATIONS OF RESPIRATORY ENZYMES IN PALE GREEN MUTANT CHLAMYDOMONAS (672)

WAVELENGTHS USED (mμ)	PROBABLE IDENTITY	ABSORBANCY CHANGES RELATIVE TO c	ΔE(cm^{-1} × mM^{-1})	CONCENTRATIONS RELATIVE TO c
430–445	a_3CO (?)	< 0.7	90	0.15 (?)
562–575	b	0.8	20	0.8
551–540	c	1	20	1
470–510	Flavoprotein	1	11	1.8
370–374	Pyridine nucleotide	2.8	6	9

absorption characteristics with respect to cytochrome a, for in the region of the α-band of this pigment, about 605 mμ, there is a scarcely distinguishable peak. Furthermore, no absorption band attributable to cytochrome a_3 appears in the Soret region at 445 mμ. Since there is no evidence in favor of a terminal oxidase of the $a + a_3$ type, other possibilities for the terminal oxidase of these algae were considered; for example, the carbon monoxide binding pigment found in so many micro-organisms (16). If the carbon monoxide binding pigment were the terminal oxidase, a distinctive absorption band would be expected to appear at 415 mμ upon the addition of CO. The dashed portion of the trace in figure 5 shows that this is not the case; no distinctive band appears at 415 mμ, in fact, the absorbancy changes for CO addition are very nearly identical to those caused by nitrogen. There is, however, a small diminution of the intensity of the absorption band in the region of 445 mμ and a slight intensification in the region of 425 mμ. These effects suggest the presence of cytochrome a_3-CO (16). Thus, the small effects in the region of 615 mμ in the oxidized minus reduced spectrum, and at 445 mμ and 425 mμ in the oxidized minus carbon-monoxide-treated spectrum suggest the presence of a terminal oxidase system more nearly similar to cytochrome $a + a_3$ than to the CO-binding pigment. An unequivocal test of this conclusion would require a study of the photochemical action spectrum for relief of CO-inhibited respiration in these micro-organisms according to the method of Castor and Chance (17), but it is unlikely that this can be carried out satisfactorily because of the rapid oxygen evolution caused by illumination of the mutant.

It has been customary in studies of various cell types to represent the components of the respiratory chains as a sequence of optical density changes relative to a particular member of the chain, and also as a sequence of concentrations. This has been done for the pale green cells and the results are given in table II. The relative concentrations of cytochromes b and c are about equal as has been observed in many other types of intact cells (18), but pyridine nucleotide and flavoprotein are observed in somewhat lower concentrations relative to cytochrome than in other cells; for example, ratios of 20 and 3, respectively, are observed in isolated mitochondria. A partial explanation for the low concentration of pyridine nucleotide relative to cytochrome is that the spectroscopic measurements are based on the transition from the aerobic steady state to the anaerobic steady state, and it is probable that only a portion of the pyridine nucleotide is affected by this transition; substrate-free cells are desirable for measuring the total concentration of pyridine nucleotide.

The relative concentration of cytochrome a_3, as assayed from the very small spectroscopic effects caused by the addition of carbon monoxide, is so low that it is questionable as to whether this component would function together with the other cytochromes as a part of the respiratory chain. In other cells the relative concentration of cytochrome a_3 to c has never shown such a disparity as has been observed here. We conclude, therefore, that it is unlikely that we have yet identified the terminal oxidase of these cells.

SPECTROSCOPIC EFFECTS OF ILLUMINATION: A typical record illustrating the effect of light compared with that of oxygen upon cytochromes of type b is given in figure 6. The record begins after anaerobiosis of the cell suspension has been established. The cells are then illuminated and an absorbancy decrease

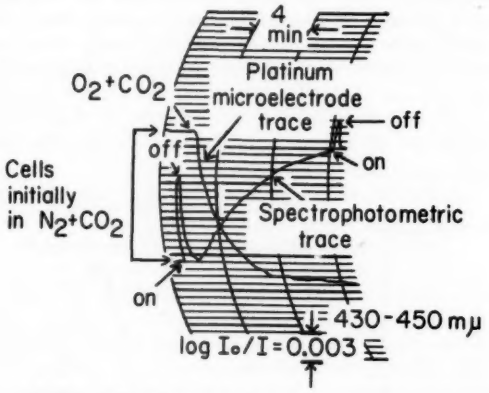

FIG. 6. The effects of light and oxygen concentration upon the absorbancy of the Chlamydomonas mutant suspension. A cell suspension, the difference spectrum of which is given in figure 7, is illuminated with red light at the moment marked "on" and the illumination is terminated at the moment marked "off." Such illumination causes an upward deflection of the trace which corresponds to a decrease of absorbancy at 430 mμ with respect to 450 mμ. The increase of oxygen concentration on changing the gas stream from $N_2 + CO_2$ to $O_2 + CO_2$ is indicated by the platinum micro-electrode trace. The corresponding oxidation of the cytochromes of the cells is indicated by the upward deflection of the spectrophotometric trace which corresponds to a decrease of absorbancy at 430 mμ with respect to 450 mμ. Illumination of the aerobic algae gives a small, further decrease of absorbancy at 430 mμ. (669 c).

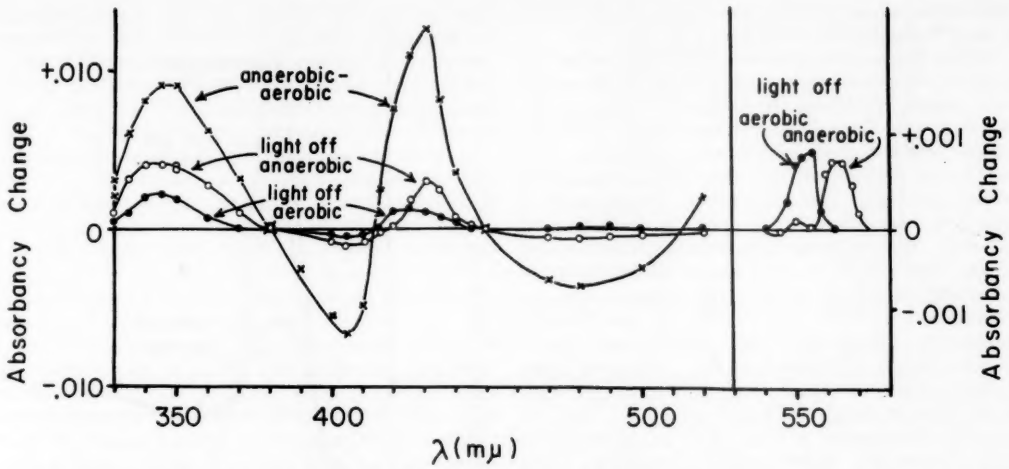

FIG. 7. Effect of aerobic and anaerobic illumination of the Chlamydomonas mutant compared with the effects of oxygenation. Repetitions of experiments similar to those of figure 6 give the difference spectra for aerobic and anaerobic light effects, as well as the aerobic minus anaerobic difference spectrum. The absorption peaks shown clearly are those due to pyridine nucleotide in the ultra-violet region and cytochromes in the region of the Soret band. In the visible region, only the aerobic and anaerobic light effects are shown because the amplification of the scale is such that the oxidized minus reduced spectrum would be far off scale. The color filter combinations appropriate to the wavelength regions are discussed in the text. (673).

occurs as is indicated by the upward deflection of the spectrophotometric traces. This decrease corresponds to an oxidation of cytochrome b. On cessation of illumination, the absorbancy increases towards the anaerobic value. The cells are then oxygenated with a mixture of O_2 and CO_2 as is indicated by a downward deflection of the patinum electrode trace and the absorption of 430 mμ decreases as the oxygen concentration increases. After the cells have been oxygenated, illumination causes a smaller absorbancy decrease. Thus an exhaustive study of the light effect requires recordings of the spectra in both the aerobic and the anaerobic states. Figure 7 illustrates such an experiment and, in addition, gives for comparison the oxidized minus reduced spectrum for the region of 330 mμ to 520 mμ. The absorbancy changes are plotted with the aerobic state as the base-line for the oxidized minus reduced spectrum and the dark state as the base-line for the light-on-light-off spectrum. Thus, the congruence of the traces shows that oxygenation and illumination or nitrogenation and darkening cause similar oxidation-reduction changes.

In the case of pyridine nucleotide, illumination of the anaerobic cells causes about half the oxidation that occurs with oxygenation. Nevertheless, illumination of the aerobic cells causes a further small increase of oxidation of pyridine nucleotide.

Studies have also been made on the effect of red illumination upon absorbancy changes in a Chlorella suspension placed in the moist chamber in the manner described above, other details of the experimental technique being approximately the same as those described above. In Chlorella the absorption of the material in this region of the spectrum is so large that the amount of measuring light falling on the phototube is small compared to the leakage of the red light through the filter combination. As shown in figure 8, illumination causes a transient downward deflection and cessation of illumination causes a transient upward deflection. These deflections represent the response of the resistance-capacitance coupling circuit of the amplifier to the square pulse of photocurrent caused by turning on and by turning off the light and do not represent spectroscopic changes. Following this transient deflection, true spectroscopic effects can be recorded and an absorbancy decrease at 340 mμ is observed upon illumination. Upon turning off the light, the absorption increases towards the initial level and the phenomenon may be repeated upon illuminating for a second time. Thus, in Chlorella we find

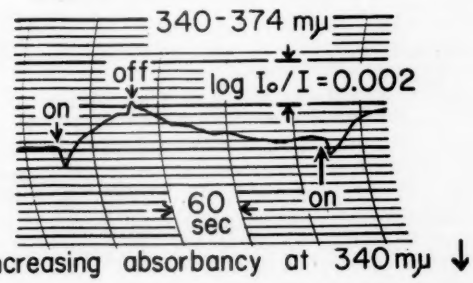

FIG. 8. The absorption spectra changes caused by red light illumination of the Chlorella suspension. For details see (6). (623 e).

confirmation of the phenomenon demonstrated clearly in the Chlamydomonas mutant.

While illumination causes qualitatively similar effects in both aerobiosis and anaerobiosis in the ultraviolet region, the effects are distinctly different in the region of the Soret band. Illumination under anaerobic conditions primarily affects cytochrome b as seen by the peak at 430 mμ, while illumination under aerobic conditions primarily affects cytochromes of type c or f as shown by the peak in the region of 425 mμ. The difference between the anaerobic and aerobic light effects is more clearly distinguished in the visible region of the spectrum where peaks attributable to cytochrome b at 563 mμ and to cytochrome f at 555 mμ are found. Flavoprotein, interestingly enough, although it shows a relatively large change of absorption in the oxidized-reduced spectrum, shows little or no effect upon illumination. This remarkable discrepancy is made clear by reference to figure 9, especially under anaerobic conditions where such a large oxidation of cytochrome b is observed. Effects upon cytochrome a_3, if they occur, are too small to measure.

It is found that the effect of light compared with that of oxygen varies with the characteristics of the cell suspension (possibly age and endogenous substrate content) and figure 9 represents a suspension which showed a very large anaerobic light effect. In fact, the oxidation of cytochrome b caused by illumination very nearly equals that caused by oxygen. This spectrum also clearly shows the distinction between aerobic and anaerobic light effects both in magnitude and in position of the peak; the Soret peak of the anaerobic light effect lies 4 mμ below that of the aerobic effect.

The data of both figures 7 and 9 indicate that no measurable absorbancy change has occurred at 515 mμ, a wavelength at which distinctive effects are observed in algae containing their full complement of pigments. Since these organisms carry out the photosynthetic process, the lack of the 515 mμ absorption band is of some significance.

The rather distinctive effects of oxygen upon the response to illumination at 554 mμ and 563 mμ is illustrated by figures 10 A and 10 B, respectively.

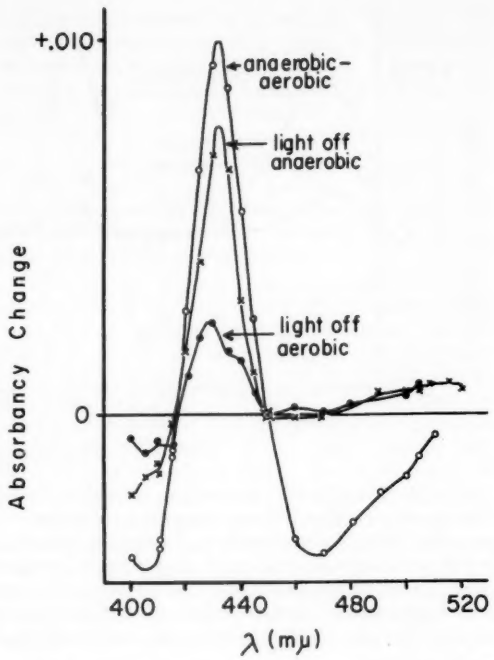

FIG. 9. Light and oxygen effects in an algal suspension in which the anaerobic light effect nearly equals the effect of oxygen. The difference spectra are obtained by repetition of experiments with the cell suspension of figure 6. Noteworthy is the very small effect of light upon the oxidation-reduction state of flavoprotein measured in the region of 470 mμ. (669 c).

These are the direct photographic recordings of the spectrophotometer output when the monochromators are set at wavelengths 554 and 540 mμ in figure 10 A and 563 and 540 mμ in figure 10 B. The trace begins with the cells in the aerobic state. Upon illumination the upward deflection indicates the decrease of absorbancy at 554 mμ. This absorbancy decreases rapidly and returns to the initial level upon cessation of

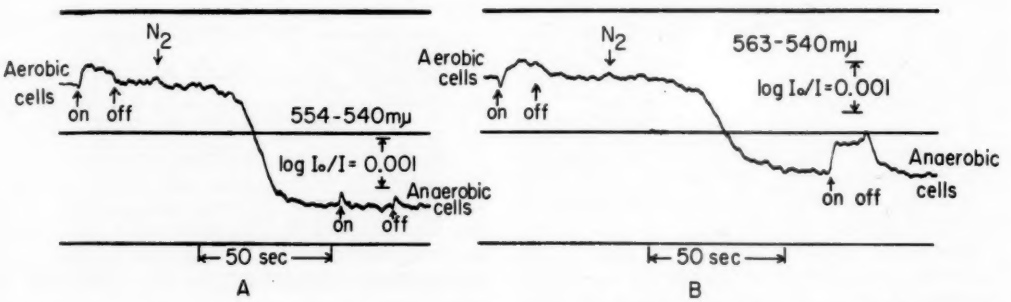

FIG. 10. The kinetics of the effects of illumination and disoxygenation measured at wavelength appropriate to cytochrome f (fig 10 A) and cytochrome of type b (fig 10 B). Other details of the experiment are included on the figure. (669 c).

the illumination. The gas passing through the moist chamber is then changed to nitrogen and the trace shows the absorbancy increase caused by the reduction of the cytochrome. Some seconds after anaerobiosis has been established, illumination is repeated and no significant change other than a brief transient is recorded. The record at 563 mμ differs in that there is a slight response to illumination in the aerobic state and a larger increase in anaerobiosis. These records also give some indication of the clarity of recording of these small absorbancy changes in the visible region of the spectrum. The noise fluctuation of optical density is less than 10^{-4} and yet the speed of response is considerably less than 1 second.

A detailed spectrum of the effects in the visible region is shown in figure 11. The anaerobic light effect shows the distinctive and sharp peak at 563 mμ. The subsidiary peak lies very close to 554 mμ (this peak shows somewhat less clearly in figure 7 and its magnitude relative to that of 563 mμ depends upon the interval of illumination as illustrated by figure 12 below). The aerobic light effect has a peak around 556 mμ which is the appropriate wavelength for cytochrome f.

KINETICS OF THE ANAEROBIC LIGHT EFFECTS: Figure 10 A showed that no appreciable absorbancy change occurs at 554 mμ for an illumination lasting a few seconds. Nevertheless, a definite peak shows in the spectra of figures 7 and 11. This is due to the longer interval of illumination used in those experiments and the two apparently divergent results may readily be correlated by reference to figure 12. This

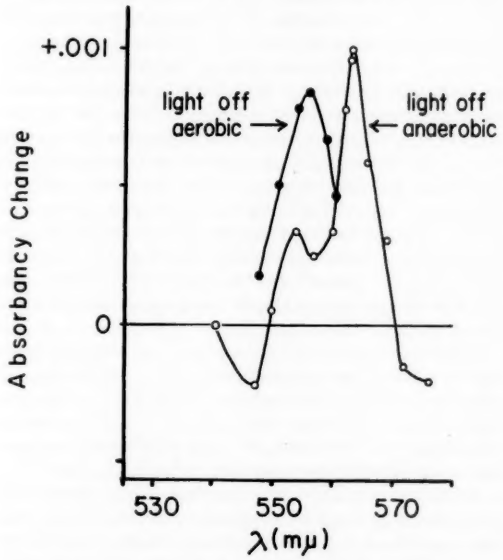

FIG. 11. A detailed study of the effects of light upon the α-bands of the cytochromes under aerobic and anaerobic conditions. The sensitivity used in this experiment is adequate to detect the small shoulder on the peak of cytochrome b in the anaerobic light effect. (674).

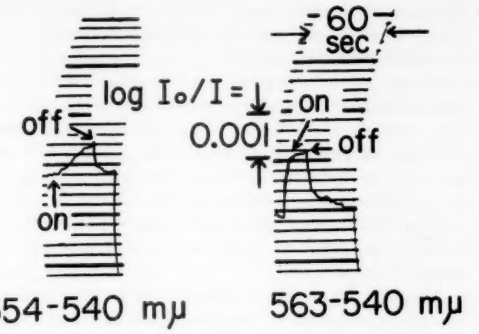

FIG. 12. A comparison of the kinetics of the light effect at wavelengths appropriate to cytochrome f (*left*) and cytochrome of type b (*right*). The same cell suspension was used in this experiment as in the experiment of figure 10. (674).

figure shows absorbancy changes in response to long period illumination of the anaerobic suspension. The typical rapid response measured at 563 mμ is shown in figure 12 and this agrees with that of figure 10 B which shows this response to occur in less than a second. The response measured at 554 mμ is much slower; immediately upon illumination there is no response at all (cf fig 10 A). After 30 seconds illumination, a considerable absorbancy decrease has occurred. Upon cessation of illumination, this change diminishes abruptly. Thus, the behavior of the two cytochromes upon illumination differs, that of type b reaches its steady state and returns therefrom symmetrically, while cytochrome f shows a lag in response to illumination of the anaerobic cells and a rapid response to cessation of illumination.

DISCUSSION

As stated in the introduction, there is little consistency of the various experimental studies on the spectroscopic changes caused by illumination of the green cell. Nevertheless, various conclusions have been drawn, and we propose to examine such conclusions in the light of the data obtained in these experiments.

1. THE OXIDATION OF CYTOCHROME f UPON ILLUMINATION: In Duysens' first paper he attributed a decrease of absorption based on a single point at 420 mμ to the oxidation of cytochrome f upon illumination of Chlorella (3). More points were obtained which confirmed the existence of such a peak at 420 mμ in later work on *Porphyridium cruentum* (4). However, 424 mμ is the correct location (see footnote 3) for the peak of the Soret band of cytochrome f (19), and Duysens' data can therefore be used only to support the supposition that cytochrome c is oxidized upon illumination of both Chlorella and Porphyridium. In Porphyridium, however, Duysens' data show the disappearance of a 555 mμ peak on illumination which he attributes to cytochrome f oxidation, and this is the correct wavelength for the band of cytochrome f (19).

The data of Strehler and Lynch (7), taken from the "negative overshoot" phase of their spectroscopic record, suggest that cytochrome f is *reduced* upon illumination. An alternative interpretation of their results, which is in no way inconsistent with the data they present, is that the "negative overshoot" phenomenon applies only to the 518-mμ band and does not apply to the 555-mμ band. We suggest that they were observing an oxidation of cytochrome f that persisted about 1 second after cessation of illumination, a supposition that is not ruled out by our data on cytochrome f (cf fig 10).

Lundegårdh (8) states that he measured an oxidation of cytochrome f "at illumination," but due to his slow recording method this result must (a) depend upon an extremely long persistence (30 seconds) of the oxidation of cytochrome f upon the illumination of Chlorella or (b) be related to indirect effects of illumination (see footnote 4). That the spectroscopic effect persists at any reasonable magnitude for approximately 30 seconds is a result contrary to our records of cytochrome f kinetics (see fig 10). Thus, it may only be a fortuitous circumstance that Lundegårdh's data agree with other experimental results.

In summary, Duysens showed that both cytochrome c and cytochrome f are oxidized upon illumination under his experimental conditions, a re-interpretation of Strehler and Lynch's result is also in favor of oxidation of cytochrome f, and the confirmation afforded by Lundegårdh's results remains of dubious value in view of the large interval between illumination and measurement. The results of this paper show that cytochrome f is oxidized upon illumination under aerobic conditions.

The lack of a measurable oxidation of cytochrome f under anaerobic conditions can be attributed to two factors: 1) that relatively more reductant is present anaerobically than aerobically and 2) that cytochrome of type b has a higher affinity for the oxidant than f. Both these factors are consistent with the considerable delay between the oxidation of cytochrome of type b and cytochrome f that is illustrated by figure 12. It is possible that Duysens' oxidation of cytochrome c occurred under anaerobic conditions, especially in his studies of Porphyridium where he allowed the cell suspension to stand "about half a day" (4) in order to enhance the spectroscopic effects.

2. The Oxidation of Cytochrome of Type b: It is not possible for us to determine which one of the three possible cytochromes of type b is actually under observation because of the similarity of their absorption bands (cytochromes b_6, b_3, and b of the respiratory chain), and our observations suffer from the same limitations as those of Hill on the leaves of golden varieties of certain plants. In such leaves, Hill observes cytochrome b_6 to be completely reduced even in the aerobic illuminated leaf. Our results on Chlamydomonas mutant show that the cytochrome of type b can show only a small further oxidation under aerobic conditions, suggesting that it is already largely oxidized, especially under illumination. Thus, these observations differ from those of Hill and do raise questions about the magnitude of the oxidation-reduction span between cytochrome of type b and cytochrome f in the illuminated cell. Our results suggest that both cytochromes of type b and cytochrome f are more oxidized in the aerobic illuminated green cell.

3. The Oxidation of Reduced Pyridine Nucleotide under Aerobic and Anaerobic Conditions: Duysens' experimental data, from which he has drawn the conclusion that pyridine nucleotide is reduced upon illumination of Chlorella and Porphyridium (4), are indistinct and are contrary to the results obtained here. His recordings cover the range from 370 to 320 mμ and show a general increase of absorbancy in this region upon illumination. When his curves are studied in detail, however, it is difficult to see how this general increase of absorption could be attributed specifically to the reduction of pyridine nucleotide. For example, his curve for Chlorella clearly shows the absorption at 350 mμ to exceed that at 340 mμ; the reverse is true for authentic DPNH. In the case of Porphyridium, the data show a small peak at 340 mμ but there is an equally large peak at 360 mμ. A part of this change could be attributed to the δ-band of cytochrome f which gives an absorbancy change upon illumination that is larger at 360 than at 320 mμ. Thus, the spectroscopic evidence does not support Duysens' interpretation in favor of pyridine nucleotide reduction. On the other hand, Duysens' data surely did not show a change of absorbancy that could be attributed to an oxidation of reduced pyridine nucleotide, and there is at the present time no explanation of the discrepancies between his results and ours. In our experiments on Chlorella at 340 mμ, the net effect of illumination was a decrease of absorbancy at 340 mμ measured with respect to 374 mμ (fig 8), indicative of an oxidation reaction. In the Chlamydomonas mutant, clear-cut results in the region from 330 to 370 mμ were obtained upon illumination under aerobic and anaerobic conditions. This result is adequately controlled by the response of the anaerobic cells to aerobiosis, i.e., the effects of illumination show the same sort of absorption maximum and are of magnitude that is consistent with the effect of oxygen. Thus Duysens' theory (4) that oxidized pyridine nucleotide reacts with excited chlorophyll to give reduced pyridine nucleotide lacks support.

One explanation of the oxidation of reduced pyridine nucleotide under aerobic conditions that is to be considered is that light-induced oxygenation of the interior of the cell causes further oxidation of pyridine nucleotide. If this were so, other respiratory components should also show further oxidations. Actually, cytochrome f is the only one that shows distinctive changes under aerobic conditions and cytochrome f is not considered to be a component of the respiratory chain. Thus, the pyridine nucleotide oxidized upon illumination of the aerobic cells is probably not that associated with the respiratory chain but with the photosynthetic mechanism of the chloroplasts. This result now appears to be opposed to the chemical

studies of the effect of illumination upon isolated chloroplasts; for example, Ochoa and Vishniac (20) used enzymatic methods for demonstrating pyridine nucleotide reduction upon illumination of chloroplast fragments, and more recently, San Pietro and Lang have obtained spectrophotometrically detectable amounts of reduced pyridine nucleotide at very high DPN and chloroplast concentrations (21). One suggestion that is compatible with both types of results is that both oxidation and reduction of pyridine nucleotide occur simultaneously in the whole cell and that the reduction reaction is completely overbalanced by the oxidative one. One must postulate that the chloroplast fragments are deficient in the oxidation reaction. In any case, the fact that the net effect of illumination of the aerobic photosynthesizing cell is an oxidation and not a reduction needs to be carefully considered in mechanisms of photosynthesis.

Both Strehler (28) and Duysens and Sweep (29) have attempted to use fluorimetric methods to demonstrate DPN reduction in Chlorella, but no measurable effects are obtained on red illumination of the cells under conditions suitable for photosynthesis. However, Duysens suggests reduction can be demonstrated in *Rhodospirillum rubrum*, although a discrepancy between this result and that of Chance and Smith (10) is apparent.

4. Effect of Illumination upon the Steady State Oxidation–Reduction Level of Flavoprotein: Whereas oxygenation of the anaerobic cells causes a large oxidation of flavoprotein as evidenced by the trough at 460 mμ in the oxidized minus reduced spectrum, there is a striking lack of effect of illumination on flavoprotein under both aerobic and anaerobic conditions. While this result might have been expected under aerobic conditions, as a portion of the flavoprotein might be auto-oxidizable, it certainly is not to be expected under anaerobic conditions where the flavoprotein associated with the respiratory chain is shown to be considerably reduced in other cells, and would be expected to be oxidized upon illumination together with the cytochromes. A working hypothesis is therefore proposed, that flavoprotein is a receptor of reducing equivalents and hence the lack of measurable changes in the steady state oxidation-reduction level of this component is due to the combined action of reducing power derived from the photolysis reaction and oxidizing power derived from photolysis and the oxidation of other respiratory components. If this hypothesis is applied to both pyridine nucleotide and to flavoprotein, the effect of reducing equivalents upon the latter is much greater.

A related effect of reducing equivalents may be found in the lag in the oxidation of cytochrome f upon illumination of the anaerobic cells, such as illustrated in detail by figure 12. It should be noted that the oxidation of cytochrome f proceeds slowly and reaches its steady state value after an illumination interval of about half a minute. On cessation of illumination, the reduction of cytochrome f proceeds very rapidly. This would suggest that cytochrome f is being oxidized against a preponderance of reducing substances which have accumulated during the dark interval. We have no explanation for the fact that similar kinetics have not yet been found in the response of flavoprotein and pyridine nucleotide to illumination under aerobic conditions.

5. The Role of Carotenoids in Photosynthesis in the Mutant Cells: Three results of this paper bear upon the participation of carotenoid in photosynthesis in the mutant cell. They are:

1) that the turnover number of chlorophyll in the mutant cell is higher than that of the normal cell in spite of a β carotene content relative to chlorophyll that is 1/30th that of the dark-grown normal cell;

2) that β carotene would have to turn over about 100 times faster in the mutant cell than in the normal to keep pace with the photosynthetic activity;

3) that the action spectrum for photosynthetic oxygen evolution shows a peak due to chlorophyll, but no detectable shoulder due to carotenoid, as would be expected if it participated in the activation of chlorophyll by light;

4) that the 515-mμ absorption band does not appear upon illumination of the mutant cells. It has been proposed elsewhere that this band is due to a compound of carotenoid and, on this basis, the lack of the 518-mμ band is consistent with a lack of carotenoid participation. Since about equal amounts of chlorophyll were used in experiments with normal and mutant cells, the 30-fold deficiency of β carotene relative to chlorophyll in the mutant cells would not be sufficient to prevent the detection of a 515-mμ absorption band upon illumination.

These data indicate that the mutant cells actively photosynthesize without any physical evidence for the participation of the small amount of carotenoid that they contain. A possible function of carotenoid is discussed below.

These studies with the mutant also shed considerable light on the nature of the chemical change that could give rise to the 518-mμ absorption band in the normal cells. From an observation of several components of the respiratory chain, it is shown here that the predominant effect of illumination of the aerobic or anaerobic cell is an oxidation reaction; reduction reactions can be identified only by a lag period in an oxidation of cytochrome f or the absence of an oxidation of flavoprotein: no direct reductions have been observed of any components of the whole cell. The presence of an oxidant and the lack of a reductant supports the hypothesis presented in the paper of Chance and Strehler (6) that the 518-mμ absorption band (phase 3 reaction) (6) results from an oxidation reaction rather than a reduction reaction.

Further evidence for the oxidation reaction may be inferred from the growth conditions for both this mutant and that of *Rhodopseudomonas spheroides;* they are sensitive to prolonged illumination to such an extent that the cells are killed. It is possible that the appearance of the 518 mμ absorption band in the normal type Chlamydomonas or in Chlorella is an in-

dication of a protective reaction in which excess oxidizing equivalents produced by the photosynthetic process react with a pigment, presumably carotenoid, in order to prevent damage to the cell, and such a hypothesis has recently been presented on the basis of work with *R. spheroides* (23). The possible nature of the reaction was discussed by Calvin (24) and it is probable that the oxidant involved is not molecular oxygen but rather an intermediate in the photolysis of water, since neither the Chlamydomonas nor the *R. spheroides* mutant is sensitive to oxygen itself.

6. Position of Cytochromes b and f in the Sequence of Illumination Reactions: The much more rapid oxidation of cytochrome b than f under anaerobic conditions in response to the illumination of the anaerobic cells suggests the manner in which these two cytochromes may react with the oxidizing and reducing equivalents produced by the photolysis of water. The reaction kinetics suggest that the oxidizing equivalents are received first by cytochrome b and then, with considerable delay, by cytochrome f. But such a sequential action of cytochromes b and f is unlikely in view of the difference in their oxidation-reduction potentials and it is possible that they are oxidized by different systems; b by the terminal, or oxygen, oxidase, and f by photolysis intermediates. A plausible hypothesis is that the oxidizing systems affect cytochromes b, and, under anerobic conditions, f, but f can respond only slightly because it is already reacting with reducing equivalents. Under aerobic conditions, cytochrome b responds only slightly to illumination, since it is already supplied with adequate oxidizing power from the terminal oxidase under these conditions. Under these conditions, the effect of light upon cytochrome f is rapid, presumably because less reducing power is present, for example, less reduced pyridine nucleotide is available (see fig 7) cytochrome b is already oxidized. Such a reaction sequence is in no way in accord with the oxidation-reduction potentials of the isolated pigments from which the opposite results would have been expected upon their reaction with oxidizing equivalents. Whether the cytochromes in the intact cell have different oxidation-reduction potentials from those obtained upon isolation, or whether some unknown factor is affecting the reaction kinetics is a point which cannot be decided at the present time, and further studies are needed.

7. Pathways for Respiratory and Photosynthetic Electron Transport and Phosphorylation: Since the components of the respiratory chain observed in the oxidized minus reduced spectrum for the Chlamydomonas mutant resemble, with the exception of the terminal oxidase, those of mitochondria capable of oxidative phosphorylation, we can presume that the respiratory chain of the mitochondria of the green cell consists of a similar series of components:

$O_2 \rightarrow$ oxidase $\rightarrow$ cytochrome c $\rightarrow$ cytochrome b $\rightarrow$ flavoprotein $\rightarrow$ pyridine nucleotide $\rightarrow$ substrate

While Hill has proposed that cytochrome f is more likely to react with the photo-produced oxidant than is cytochrome b, the delay in the oxidation of cytochrome f observed upon illumination of the anaerobic cells together with the rapid response of cytochrome b must be taken into consideration in any mechanism for photosynthetic electron transport and phosphorylation. Our data on flavoprotein suggest that this component is nearest to the source of the reducing equivalents. At the present time our inability to discriminate between components of the respiratory and photosynthetic chains is limited by the fact that our observations are based upon the whole cell. Further experiments on particles isolated from these cells will be necessary to distinguish between the enzymes that are involved in the pathways of photosynthesis and respiration.

Summary

Utilizing senstive spectrophotometric techniques and a pale green mutant of Chlamydomonas which carries out an active photosynthesis with a low chlorophyll content, the components of the respiratory chain have been investigated. It is found that pyridine nucleotide, flavoprotein and cytochromes b and c are present in amounts typical of other micro-organisms. The terminal oxidase is present in such a low concentration relative to the other cytochromes that it has not been surely identified.

Pyridine nucleotide is affected by light aerobically and anaerobically; flavoprotein is scarcely changed under either condition. Cytochrome b responds most sensitively to illumination anaerobically but responds very little to illumination aerobically. Cytochrome f responds rapidly to illumination under aerobic conditions and sluggishly under anaerobic conditions. No evidence of increased absorption at 518 $m\mu$ upon illumination is recorded. Since the mutant has a high photosynthetic activity per unit chlorophyll content, it is probable that the compound absorbing at 518 $m\mu$ and observed upon illumination of the normal cells is not an essential intermediate in photosynthesis.

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MALONYLTRYPTOPHAN IN HIGHER PLANTS[1,2]

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During the course of our study of indoleacetic acid metabolism (1, 6), we investigated the effect of exogenous tryptophan on the synthesis of indoleacetylaspartic acid in excised pea epicotyls. Tissues bathed for 24 hours in solutions containing 50 to 200 mg/l tryptophan accumulated considerable amounts of a substance which closely resembled indoleacetylaspartic acid in chromatographic mobility in several solvents, in acid strength, and in color reactions with the Ehrlich (*p*-dimethylaminobenzaldehyde) and Salkowski (acid-ferric chloride) reagents (fig 1). Consequently, in discussing metabolic precursors of indoleacetic acid at the 1956 Annual Meeting of the American Society of Plant Physiologists at Storrs, Connecticut, we reported erroneously the conversion of tryptophan into indoleacetylaspartic acid. Differences soon became apparent. The unknown substance, which occurred in several families of higher plants, had a slightly higher R_f in most solvents than had indoleacetylaspartic acid. Furthermore, although the color produced on paper with the Salkowski reagent was the purple of indoleacetylaspartic at very low concentrations, the color at higher concentrations was brown; in this respect the substance resembled acetyltryptophan. Basic hydrolysis of the unknown yielded tryptophan, not indoleacetic acid.

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Isolation

In order to assign a structure to the tryptophan derivative its isolation in a relatively pure state was necessary. This isolation was a somewhat laborious procedure since the solubilities of the derivative were similar to those of the bulk of the plant acids. However, it was possible to take advantage of the fact that it was a very strong acid and a considerable purification was achieved by partitioning the plant

acids between water and *n*-butyl alcohol, or water and ether, with careful pH regulation; fortunately it was a simple matter to determine, rapidly and quantitatively, the distribution of the tryptophan derivative between the organic and aqueous phases by use of the above mentioned Ehrlich reagent.

The methods ultimately adopted were as follows: two kilograms of spinach leaves, purchased at the grocers', were incubated with a tryptophan solution (100 mg/l) in large crocks oxygenated with a stream of air bubbles. A coarse screen and weights kept the leaves submerged. After 24 hours the fully turgid and apparently healthy leaves were removed, washed, ground in a Waring blendor with a little water and enough sodium bicarbonate to make the brei 0.25 M. The brei was saturated with ammonium sulfate and the aqueous phase was removed by filtration on a Büchner funnel with the aid of a generous amount of infusorial earth. The filtrate, which was free of chlorophyl and quite clear, was acidified to pH 4.3 with phosphoric acid and extracted four times with ether. This ether was discarded. The filtrate was then acidified again, this time to pH 2.5 and extracted 5 times with ether. The extract was made alkaline with a few drops of concentrated ammonium hydroxide solution and the ether was removed on a steam bath in a stream of air. The residue was taken up in about 100 ml of 0.25 *N* sodium bicarbonate solution. The bicarbonate solution was acidified to pH 5.6 and extracted twice with 15-ml lots of *n*-butyl alcohol. This butyl alcohol was set aside for reworking. The aqueous phase was further acidified to pH 3.2 and extracted four times with *n*-butyl alcohol in 15-ml lots. This time the alcohol phase contained most of the tryptophan derivative. The butyl alcohol was extracted three times with 0.25 *N* sodium bicarbonate solution (total volume 70 ml). The bicarbonate solution was acidified to pH 4.3 and extracted 3 times with 30 ml of ether. This ether was set aside for reworking. Then the aqueous phase was further acidified to pH 2.5 with phosphoric acid and extracted 6 times with ether. The aqueous phase was set aside for reworking and the ether phase, containing about half the original tryptophan derivative, was taken almost to dryness in an air stream (heat avoided).

The residue was taken up in a small volume of alcohol, transferred as streaks to four sheets of Whatman No. 1 filter paper (46 × 38 cm) and chromatographed in an isopropyl alcohol–concentrated ammonium hydroxide–water solvent (80 : 10 : 15, v/v). The tryptophan derivative was located by spraying strips from the developed chromatograms with the Ehrlich reagent (1 % *p*-dimethylaminobenzaldehyde dissolved in equal volumes of alcohol and concentrated HCl). The appropriate regions were then cut out as carefully as possible using the fluorescence of various contaminants in ultra-violet light as precise markers. In order to achieve maximum purification a considerable loss was deliberately introduced in this operation. The tryptophan derivative was eluted from the paper with 1 *N* ammonium hydroxide solution. The eluate was acidified to pH 2.5 with phosphoric acid and extracted 12 times with ether. The ether was then removed in an air stream as above and the chromatographic procedure was repeated twice more—first in a solvent consisting of pyridine, *t*-butyl alcohol and water (35:35:30, v/v) and then in the alcoholic phase from a mixture of *n*-butyl alcohol, acetic acid and water (80:20:10, v/v). The final chromatogram was also eluted with 1 *N* ammonium hydroxide solution and the eluate was again acidified to pH 2.5 and extracted with ether. The tryptophan derivative, about 40 mg, crystallized out of the water residue left after removal of the ether.

Identification

These crystals melted about 80° C, were slightly deliquescent and obviously impure. The ultraviolet absorption spectrum of an alcoholic solution of the crude material was almost indistinguishable from that of acetyltryptophan. Assuming that practically all the absorption at 280 and 288 mμ was due to the tryptophan part of the molecule and that the molecule contained only one tryptophan residue, it was possible to calculate a molecular weight of 306 for the compound. The impurities present in unknown amounts, obviously made this value too high—by how much we could not know. A single determination of the neutralization equivalent, using 8.3 mg of the crystals, gave a value of 144. Clearly the tryptophan derivative was a dibasic acid. The titration curve obtained on neutralization of the acid also suggested two dissociation constants, one (pK_1 about 3.2) being definitely stronger and the other (pK_2 about 4.2) being somewhat weaker than that of acetyltryptophan (pK about 3.8). From these data it was possible to predict that the unknown part of the molecule was itself a dibasic acid, molecular weight not greater than 120, attached to the amino group of tryptophan through one of its carboxyls. Moreover the acid had to be very strong because the first dissociation constant of the condensed compound (pK_1 3.2) presumably reflected the ease of ionization of the remaining free carboxyl; since the tying up of one carboxyl of a dibasic acid usually decreased the dissociation constant of the remaining carboxyl, the first pK of the parent dicarboxylic acid was presumably lower than 3.2. Very few acids could meet these requirements. In fact the only ones to do so were oxalic (pK_1 1.32, MW 90), malonic (pK_1 2.83, MW 104), maleic (pK_1 1.92, MW 116) and fumaric (pK_1 3.02, MW 116). Succinic acid (MW 118) was improbable because of its relative weakness (pK_1 4.19).

Samples of the crude crystalline material were hydrolysed in 3 *N* barium hydroxide solution in a steam autoclave at 20 lb pressure per square inch for three hours. Carbon dioxide was bubbled through the cooled and diluted hydrolysate until the excess barium had been precipitated as carbonate. The carbonate was removed by filtration and washed. The filtrate and washings were then taken to dryness and the residue taken up in 70 % alcohol containing an

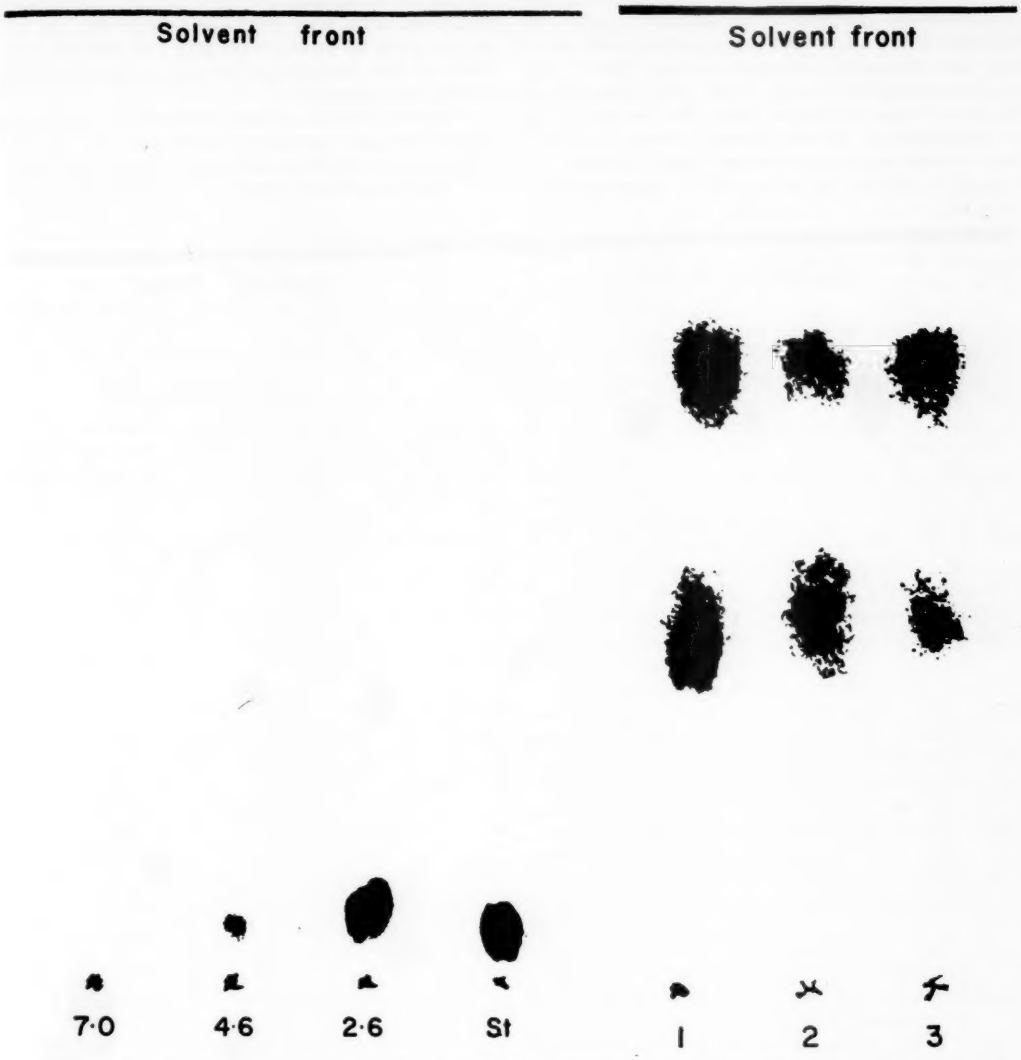

FIG. 1 (*left*). Paper chromatogram of ether extracts of tryptophan treated pea epicotyls. Developing solvent isopropyl alcohol–concentrated ammonium hydroxide–water solvent (80 : 10 : 10, v/v). The chromatogram was sprayed with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde) in alcohol-aqueous HCl. Numbers refer to the extraction pH. St. is synthetic indoleacetylaspartic acid. Extracts from 3 gm fresh weight of epicotyl tissue were applied to the chromatogram.

FIG. 2 (*right*). Paper chromatogram of malonic acid (R_f 0.65), tryptophan (R_f 0.37), and the hydrolysis products of the ether soluble tryptophan derivative from tryptophan treated spinach leaves. Developing solvent: Alcoholic phase from a mixture of *n*-butyl alcohol, formic acid and water (30 : 6 : 45, v/v). The acids were located by their inhibition of mercurochrome fluorescence. 1. A mixture of 0.7 micromoles tryptophan and 0.7 micromoles malonic acid. 2. Tryptophan derivative (approximately 0.7 micromoles) after hydrolysis with 3 *N* barium hydroxide for 3 hours in an autoclave. 3. A mixture of malonic acid and tryptophan (both 0.7 micromoles) after similar treatment with barium hydroxide.

excess of formic acid to decompose the barium salts. The hydrolysis products in alcohol were applied to Whatman No. 1 paper and chromatographed in a *n*-butyl alcohol–formic acid–water solvent (30 : 6 : 45, v/v, alcoholic phase used). Acids were detected on the dried chromatograms by their effect in preventing the fluorescence of mercurochrome (dibromohydroxymercurifluorescein) in ultra-violet light. Figure 2, a chromatogram sprayed with a 0.02 % mercurichrome solution, dried and photographed with the visible light emitted on ultraviolet irradiation, shows that the hydrolysis products were tryptophan and malonic acid. Oxalic, maleic, fumaric and succinic acids have quite different mobilities.

Oxalytryptophan, malonyltryptophan and succinyltryptophan were synthesized by the action of the acid chlorides on tryptophan suspended in dry ether (3). Rigorous identification of the products was not

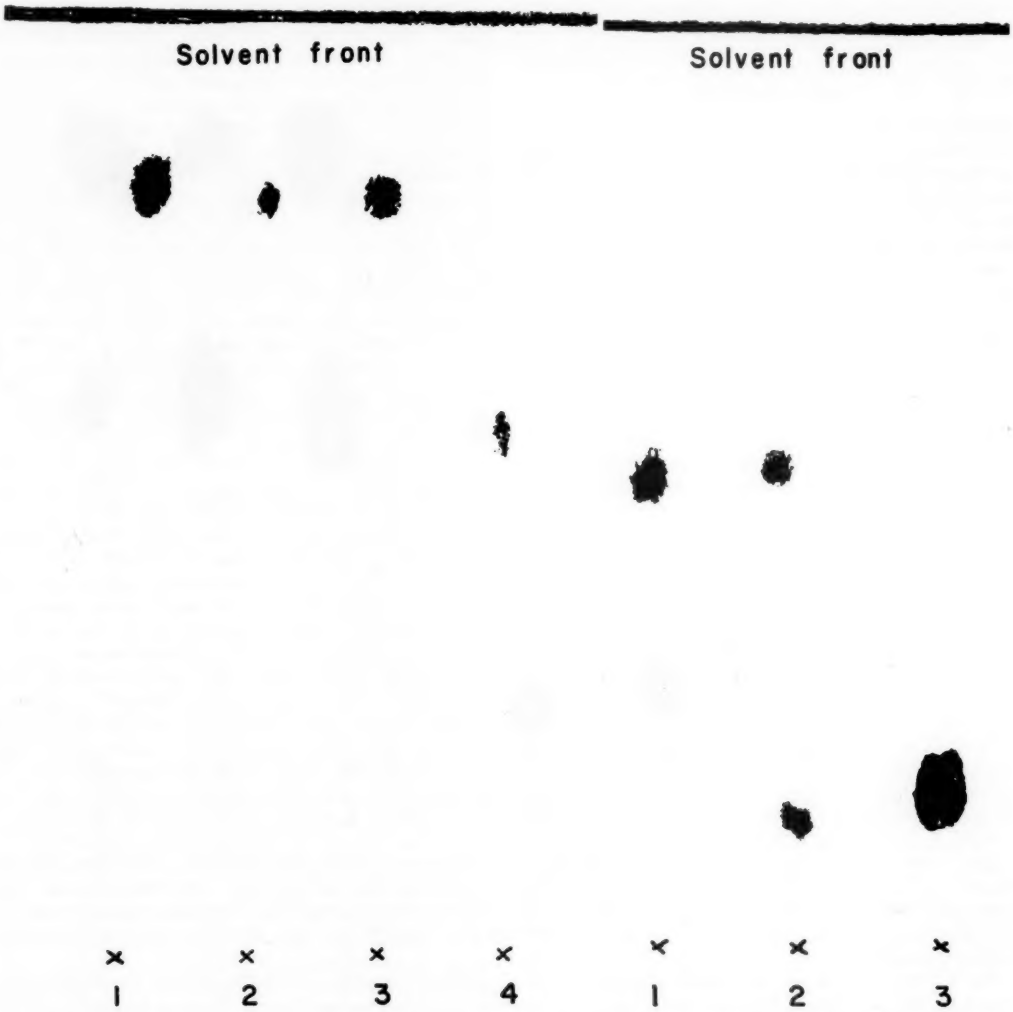

FIG. 3 (*left*). Paper chromatogram of tryptophan derivatives, developed with a solvent consisting of the alcoholic phase from a mixture of *n*-butyl alcohol, acetic acid and water (80 : 20 : 10, v/v), and sprayed with Ehrlich's reagent. 1. Synthetic succinyltryptophan. 2. Synthetic malonyltryptophan. 3. Extract of tryptophan treated spinach leaves. 4. Synthetic oxalyltryptophan.

FIG. 4 (*right*). Paper chromatogram of heated and unheated samples of the tryptophan derivative from tryptophan treated spinach leaves. Chromatogram developed with an isopropyl alcohol, ammonium hydroxide, water solvent (80 : 10 : 10, v/v) and sprayed with Ehrlich's reagent. 1. An acetyltryptophan marker. 2. Tryptophan derivative heated for about 2 minutes at 145° C. 3. Tryptophan derivative before heating.

undertaken. Malonyl chloride was prepared by the action of phosphorous pentachloride on malonic acid (4). The chromatographic mobilities of the synthetic tryptophan derivatives were compared with the mobility of the unknown in the solvents described above. In each case the unknown was very much more mobile than the oxalyl derivative, slightly but consistently less mobile than the succinyl derivative and indistinguishable from the malonyl derivative (fig 3).

Finally it occurred to us that a monoamide of malonic acid, such as malonyltryptophan, should decarboxylate on heating to yield CO_2 and acetyltryptophan. Both synthetic malonyl tryptophan and the biological material, when heated on a melting point block evolved gas at temperatures above 125° C and as can be seen in figure 4, acetyltryptophan was formed.

Physiological Significance

The malonyltryptophan observed in tryptophan treated pea and spinach tissues can scarcely have resulted from the metabolic activities of bacteria contaminating the incubation medium. Malonyltryptophan was formed consistently, regardless of the conditions of tryptophan application. In several experiments the tissues were carefully surface sterilized with NaOCl and large amounts of penicillin and streptomycin were added to the sterile incubation medium. After removal of the tissues these media were still absolutely clear. Nevertheless the usual amount of malonyltryptophan synthesis had taken place. Malonyltryptophan also seems to be widely distributed, albeit in very small amounts, in plants which have never been exposed to an exogenous source of tryptophan; consequently it is virtually certain that the plants themselves have the requisite enzyme systems for the synthesis.

A survey of the naturally occurring indole compounds in plants of various species such as tomato, spinach, pea and oats revealed substances present in small amounts in acid-ether extracts, which were chromatographically indistinguishable from malonyltryptophan, gave the same purple color with the Ehrlich reagent and brown color with the Salkowski reagent, and in the case of tomato extracts (fig 5) yielded tryptophan on hydrolysis. On the basis of the color reaction of these acid-ether extracts with the Ehrlich reagent, field grown tomato plants untreated with tryptophan contained about 0.5 mg of malonyltryptophan per kilogram fresh weight while untreated etiolated pea epicotyls contained a great deal less.

Malonyltryptophan is quite stable when laid down in the tissues. Three-week-old tomato plants were sprayed continuously with 100 mg/l of tryptophan for 24 hours in an illuminated chamber during which time they accumulated about 4 mg of malonyltryptophan per kg fresh weight or eight times the endogenous level. The plants were thoroughly washed, transferred to a greenhouse bench and entire plants were sampled every two days for 11 days. There was no decrease in the amount of malonyltryptophan per plant.

In pea epicotyls the formation of malonyltryptophan from applied tryptophan was strongly inhibited by the presence of monobasic acids such as phenylbutyric, di- and trichlorinated phenoxyacetic, napthalene acetic, benzoic and triiodobenzoic. 2,4-D or 2,6-D at 2 mg/l inhibited about 50 % of the malonyltryptophan formation and 20 mg/l inhibited the reaction completely. However, there is no evidence that the conjugation mechanism itself was inhibited since the accumulation of free tryptophan was prevented by these acids.

The authors have no idea what role malonyltryptophan plays in angiosperm physiology. Malonic acid itself seems to be rather widely found in plants often in considerable amounts (2, 5), but studies of its metabolism have been neglected. Malonic acid metabolism has been linked to acetate metabolism in bacteria by Hayaishi who showed a decarboxylation reaction involving the coenzymeA-thioesters of malonic and acetic acids as intermediates (7). If malonyl-CoA is also a metabolic intermediate in higher plants, it

Fig. 5. Paper chromatogram of concentrated ether extracts of field grown tomato plants not treated with tryptophan. Chromatogram developed with isopropyl alcohol, concentrated ammonium hydroxide, water (80: 10 : 10, v/v) and sprayed with Ehrlich's reagent. The numbers refer to the extraction pH. I is synthetic indoleacetylaspartic acid and T is a tryptophan marker. Extracts from approximately 100 gm fresh weight of tissue were applied to the chromatogram. The substance extracted at pH 2.6 (R_f 0.11) yielded tryptophan on hydrolysis.

could well be that this is the activated form of malonic acid which condenses with tryptophan. We have not yet investigated the possibility that other amino acids may be similarly conjugated with malonic acid.

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STUDIES ON 3-INDOLEACETIC ACID METABOLISM. IV. CONJUGATION WITH ASPARTIC ACID AND AMMONIA AS PROCESSES IN THE METABOLISM OF CARBOXYLIC ACIDS[1,2]

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In earlier publications it was reported that pea epicotyls convert applied indoleacetic acid into indoleacetylaspartic acid (1) and, to a much smaller extent, into indoleacetamide (6). In the present paper it will be shown that condensations with aspartic acid or ammonia are not limited to indoleacetic acid. When pea epicotyl sections were incubated in solutions of indoleformic (indole-3-carboxylic), indolepropionic, indolebutyric, benzoic, or 2,4-dichlorophenoxyacetic acids all the corresponding amides except 2,4-dichlorophenoxyacetamide, and all the corresponding aspartic conjugates except indoleformylaspartic acid, were found in the tissues.

Materials and Methods

All experiments were carried out on pea sections grown and treated under conditions previously described (6). In brief, the sections were bathed for 24 hours in M/60 sodium acid phosphate solutions containing 20 to 30 mg per liter of the acid to be investigated. The tissues were then washed and ground in a Waring blendor with sodium bicarbonate solution. The resulting brei was saturated with ammonium sulfate, infusorial earth was added and the solid matter was separated by filtration. The filtrate, about pH 7.0, was extracted several times with ether, then acidified to pH 4.6, and again repeatedly extracted with ether. The filtrate was further acidified to pH 2.6 with phosphoric acid and repeatedly extracted yet again with ether and finally with *n*-butyl alcohol. The butyl alcohol and other extracts were taken to dryness, taken up again in a small volume of alcohol and chromatographed on paper in an isopropyl alcohol, concentrated ammonium hydroxide, water solvent (80 : 10 : 10, v/v).

Identification of the metabolites was based on chromatographic comparisons with synthetic compounds and, in some cases, on the chromatographic identification of the hydrolysis products.

Indolepropionylaspartic acid, indolebutyrylaspartic acid and 2,4-dichlorophenoxyacetylaspartic acid were synthesized by the carbodiimide method as previously published (5). Benzoylaspartic acid was prepared by the action of benzoylchloride on a cold aqueous solution of aspartic acid containing an excess of NaOH. Benzamide was prepared by the action of concentrated aqueous ammonia on benzoylchloride. The amides of 2,4-D, indolepropionic acid and indolebutyric acid were prepared, conveniently, but in rather poor yields, by dehydration of the ammonium salts with dicyclohexylcarbodiimide. The derivatives of indoleformic acid were not prepared; attempts using the carbodiimide were unsuccessful. Indoleformic acid itself was synthesized as the ester by treating indole first with a Grignard reagent and then with ethylchlorocarbonate (9).

The indole derivatives were detected on the paper with the Ehrlich reagent (1 % *p*-dimethylaminobenzaldehyde dissolved in equal volumes of alcohol and concentrated hydrochloric acid). Since benzoic acid and 2,4-D do not give convenient color reactions, C^{14}-carboxyl-labeled preparations were used and the radioactive metabolites of these acids were located by preparing radioautographs of the developed chromatograms. The synthetic derivatives of benzoic acid and 2,4-D were not radioactive and were detected by exposing the developed chromatograms to iodine fumes

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[2] Contribution No. 104, Canada Department of Agriculture, Science Service Laboratory, London, Ontario.

which resulted in a brown background with lighter areas over the substances. The 2,4-D derivatives were also detected by spraying the chromatograms with riboflavin and viewing them under ultraviolet light; 2,4-D and some of its derivatives are oxidized by photoactivated riboflavin (3) with the concomitant reduction of the flavin to its non-fluorescent leuco form so that the compounds show as dark spots.

The use of radioactive benzoic acid and 2,4-D facilitated quantitative evaluation of the reactions under investigation. Samples of the ambient solution before and after the incubation period, of the incubated tissues, and of the various extracts and residues of the tissues were collected. Aliquots containing not more than 36 mg of carbon were transferred with 2 gm potassium dichromate to a boiling tube. The boiling tube was provided with a side arm and was fitted with a two-holed stopper. Through one hole sulfuric acid saturated with potassium dichromate was added while a gentle stream of CO_2-free air entered by the other hole and bubbled through the digestion mixture. This air stream then passed through the side arm into a Pettenkofer tube containing 40 ml of 2.5 % barium hydroxide solution, 3 ml of *n*-butyl alcohol (to eliminate foaming) and a few drops of phenolphthalein solution. After 25 ml of the sulfuric acid had been added to the boiling tube, the digestion mixture was slowly heated over a period of about 15 minutes until the sulfuric acid just began to reflux. Then a stream of CO_2 was passed through the apparatus until all the barium had been converted into the carbonate (disappearance of the pink color). This carbonate was transferred as a methanol slurry to planchets, dried and counted at "infinite thickness" using an end window Geiger counter.

Results

Paper Chromatography of the Indole Acids and Their Derivatives: Figure 1 (*upper*) illustrates the chromatographic behavior of the synthetic substances with the isopropyl alcohol–concentrated ammonium hydroxide–water solvent. As can be seen in figure 1 (*upper*), increasing the length of the side chain increases the solubility of the indole derivatives in the non-aqueous phase and hence their mobility. On the other hand, odd numbers of carbons in the side chain seem to increase the polarity of the compounds (compare the vastly different water solubilities of the odd and even carbon dicarboxylic acids such as malonic vs oxalic and succinic acids). Consequently indoleacetic acid is considerably more mobile than indoleformic acid (effects additive), while indolepropionic acid and its derivatives are only slightly more mobile than the corresponding acetic compounds (effects opposite). Indolebutyric acid and its derivatives are again conspicuously more mobile (effects additive).

Indoleformic acid gives a pink color with the Ehrlich reagent. All the other substances give characteristic purple colors which gradually change to blue and blue-grey on long standing.

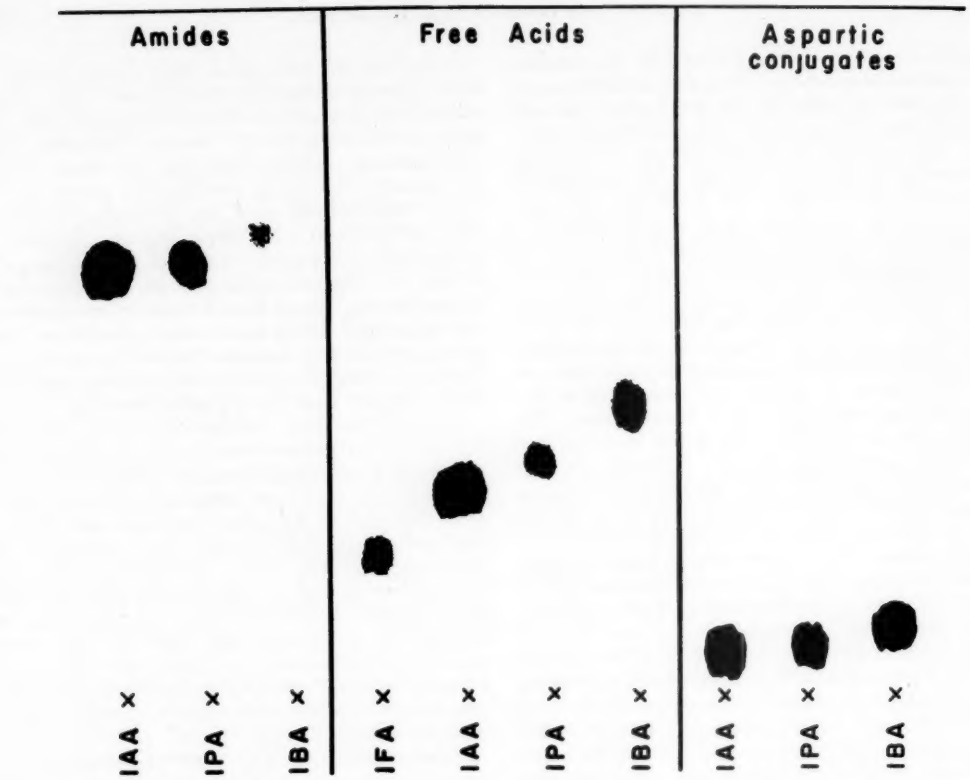
Conjugation Reactions with Indole Acids in Vivo: Chromatograms of ether extracts from tissues treated with indoleacetic acid and its homologues (30 mg/l) are shown in figure 1 (*lower*). Markers of the corresponding synthetic free acids and their amides and aspartic derivatives are included, except in the case of indoleformic acid, the derivatives of which were not prepared. The chromatogram (fig 1 B) of indoleacetic acid treated tissues has already been described in a previous publication (6) and is included for comparison. Since there were no ether-soluble indole compounds which gave a reaction with the Ehrlich reagent in ether extracts of control tissue all spots illustrated in figure 1 (*lower*) probably represent substances derived from the applied indole acids.

Indoleformic Acid (Indole-3-Carboxylic Acid) (fig 1 A): Epicotyl sections treated with indoleformic acid accumulated two Ehrlich reactive substances both of which gave the pink color associated with indoleformic acid. One of these substances was acidic and chromatographically indistinguishable from unchanged indoleformic acid (R_f 0.23). Extraction from water into ether of the second substance (R_f 0.56) was difficult; a similar solubility relation had been noted for the amide of indoleacetic acid (fig 1 B). The properties of this indoleformic acid derivative viz neutrality, relative water solubility and mobility in the isopropanol-water solvent, are those one would expect of indoleformamide.

Indoleacetic Acid (fig 1 B): Indoleacetic acid treated tissues contained, as already reported (6), indoleacetamide (R_f 0.66), free indoleacetic acid (R_f 0.35), and indoleacetylaspartic acid (R_f 0.10), as well as an unidentified spot (pH 4.6, R_f 0.27) just below IAA.

Indolepropionic Acid (fig 1 C): Pea epicotyls incubated with indolepropionic acid accumulated several indole compounds. A mobile compound (R_f 0.65) was chromatographically indistinguishable from indolepropionamide and gave the same color reaction with the Ehrlich reagent (purple) and Salkowski (acid-ferric chloride) reagent (brown). The most abundant substance (R_f 0.36) was an acid, extracted at pH 4.6, which corresponded in every respect with unchanged indolepropionic acid. A stronger acid (extractable at pH 2.6) moved more slowly (R_f 0.10) and was chromatographically similar to indolepropionylaspartic acid. This substance was hydrolyzed in 3 *N* barium hydroxide solution at 100° C for 3 hours, yielding indolepropionic acid and aspartic acid. Since the acidic substance had the same mobility and the same products on hydrolysis as synthetic indolepropionylaspartic acid, the two substances were considered identical. An unidentified spot was observed (pH 4.6) with an R_f of 0.26.

Indolebutyric Acid (fig 1 D): Tissues treated in a like manner with indolebutyric acid contained a number of indole compounds. A highly mobile, neutral substance (R_f 0.73) was chromatographically indistinguishable from synthetic indolebutyramide. An acidic substance (R_f 0.48) was chromatographically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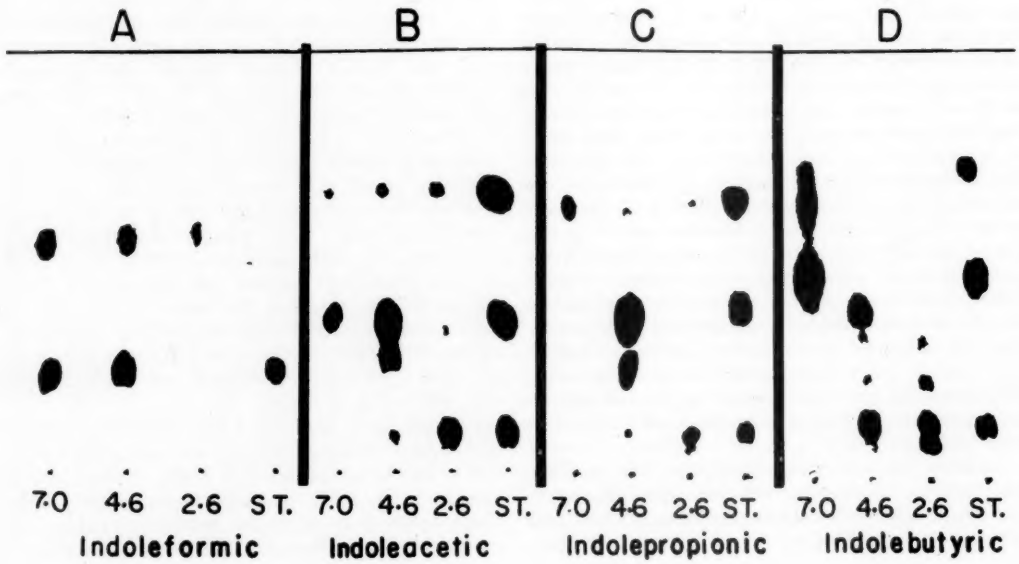
A
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Indoleformic
Indoleacetic
Indolepropionic
Indolebutyric

TABLE I

DISTRIBUTION OF THE RADIOACTIVE CARBON OF C^{14}-CARBOXYL LABELED BENZOIC ACID AND 2,4-D IN PEA TISSUES

		2,4-D		BENZOIC ACID	
		CPM	% OF ORIGINAL	CPM	% OF ORIGINAL
(A)	Original solution	85,000	100	79,400	100
(B)	Solution after incubation period	76,000	89.5	10,300	13
(C)	Lost from solution (A – B)	9,000	10.5	69,000	87
(D)	Found in tissue	6,250	7.4	21,000	26
(E)	Not accounted for. Probably lost as CO_2 (C – D)	2,750	3.2	48,100	61
(F)	Insoluble in saturated $(NH_4)_2SO_4$ solution (cell walls, proteins, etc.)	2,700	3.2	2,000	2.5
(G)	Soluble in saturated $(NH_4)_2SO_4$ solution	3,900	4.6	20,400	26
(H)	Soluble in acidified, saturated $(NH_4)_2SO_4$ solution. Insoluble in ether and *n*-butyl alcohol	165	0.2	2,650	3.3
(I)	Extracted from acidified saturated $(NH_4)_2SO_4$ solution by ether and *n*-butyl alcohol (G – H)	3,735	4.4	17,800	22

Etiolated pea epicotyl sections (10 gm) incubated in 100 ml M/60 NaH_2PO_4 solution containing 2.0 mg of labeled 2,4-D or benzoic acid (approx. 12 μc per expt).

indistinguishable from indolebutyric acid and like that acid was completely extracted by ether at pH 7.0. Two spots (pH 7.0, R_f 0.65 and pH 4.6, R_f 0.40) were not identified.

Two other substances in the indolebutyric acid treated tissues were of particular interest. These had the mobility, acid strength, and color reactions with the Salkowski and Ehrlich reagent characteristic of indoleacetic acid (pH 4.6, weak spot R_f 0.34) and indoleacetylaspartic acid (pH 2.6, R_f 0.10). Clearly, indolebutyric acid undergoes conversion, apparently by β-oxidation, to indoleacetic acid which is then conjugated with aspartic acid. There was no evidence of similar β-oxidation of indolepropionic acid to indoleformic acid.

THE METABOLISM OF 2,4-D AND BENZOIC ACID: Ten grams of pea epicotyl sections were incubated for 24 hours in 100 ml M/60 sodium acid phosphate solutions containing 2 mg of carboxyl-labeled benzoic acid or carboxyl-labeled 2,4-D (about 12 microcuries C^{14} per experiment). The radioactivity of the ambient solution was measured before and after the incubation period. Since no new radioactive compounds were detected in the bathing solutions it is probably safe to equate the disappearance of radioactivity to the amount of acid taken up by the tissues. The total radioactivity of the tissues was then determined. This was always less than the radioactivity lost from solution. Presumably the difference represents loss of carboxyl carbon in the form of CO_2. No attempt to trap the respiratory CO_2 was made, however. The tissues were ground in sodium bicarbonate solution, the brei was saturated with ammonium sulfate and the insoluble fraction, containing among other things the cellulose and precipitated proteins, was separated by filtration and washed with saturated ammonium sulfate solution. Aliquots of the soluble and insoluble materials were oxidized as described in Methods and the radioactivity of each aliquot was measured as barium carbonate. Finally the filtrate was acidified and extracted with ether and *n*-butyl alcohol and the radioactivity of the residual aqueous phase was again determined.

Table I presents the results of these determinations. Unlike indoleacetic acid (2) and benzoic acid described below, 2,4-D is not readily taken up by the pea tissue. Only 10.5 % of the 2,4-D was lost from the incubation solution. However most of this (7.4 %) was found in the tissues. Apparently during a period of 24 hours there was little decarboxylation. Of the tissue radioactivity, about 40 % was in the fraction containing the cell walls and proteins while about 60 % was in the filtrate. Acetone extraction of the cellulose-protein fraction yielded considerable amounts of 2,4-D but no other radioactive substance. The radioactivity in the filtrate was nearly all ether- or butanol-soluble and considered predominantly (over 95 %) of unchanged 2,4-D (fig 2). There was no 2,4-dichlorophenoxyacetamide (R_f 0.86). Small amounts of three other substances, all acids, were detected. The most abundant and slowest moving of these (R_f 0.15) agreed with 2,4-dichlorophenoxyace-

FIG. 1 (*upper*). Chromatograms of synthetic indoleformic (indole-3-carboxylic) acid (IFA), indoleacetic acid (IAA), indolepropionic acid (IPA), indolebutyric acid (IBA), and the corresponding amides, and the aspartic derivatives. Partitioning solvent: isopropyl alcohol, concentrated ammonium hydroxide, water (80:10:10, v/v).

FIG. 1 (*lower*). Conjugation reactions involving indole acids. Chromatograms of the ether soluble substances found in pea epicotyls which had been incubated with indoleformic, indoleacetic, indolepropionic or indolebutyric acid solutions. The numbers refer to the pH of the aqueous plant extracts shaken with ether. St. refers to standard solutions containing 1 mg/ml of the corresponding free acid, amide and aspartic acid derivatives. (For their relative positions see figure 1 (*upper*).)

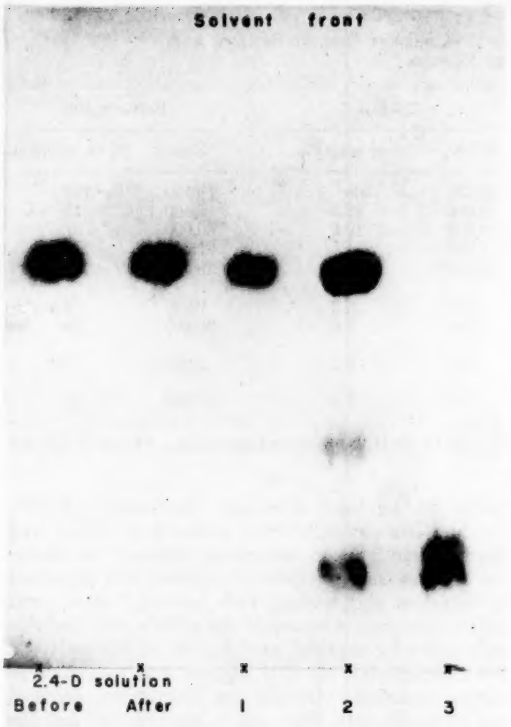

FIG. 2. Metabolites of C^{14}-carboxyl-labeled 2,4-D from pea epicotyls. Radioautographs of chromatograms of 2,4-D solutions before and after incubation and of tissue extracts: 1) ether extract at pH 7.0; 2) ether extract at pH 2.6 and 3) *n*-butyl alcohol extract at pH 2.6. The slowest moving substance in 2 and 3 is probably 2,4-dichlorophenoxyacetylaspartic acid.

tylaspartic acid in mobility and acid strength. Furthermore, this substance yielded 2,4-D on hydrolysis. The amounts involved were so small that no attempt was made to identify the aspartic acid moiety. 2,4-D which had accumulated in tissues during a 24-hour incubation did not noticeably decrease during a subsequent 24-hour period in a moist chamber.

The metabolism of benzoic acid presented a very different picture. As much as 87 % of the radioactivity disappeared from the ambient solution during the incubation period (table I). Most of this radioactivity was not in the tissues. Apparently pea tissue can readily degrade benzoic acid at least to the stage of decarboxylation. Approximately one quarter of the radioactivity lost from the solution did accumulate in the tissue, nearly all in the ether soluble fraction. Of this ether soluble material by far the greater part (90 to 95 %) was chromatographically indistinguishable from benzoylaspartic acid (fig 3, R_f 0.14) and yielded benzoic and aspartic acids on hydrolysis with barium hydroxide. Small amounts of other radioactive substances occurred. These included a trace of free benzoic acid and somewhat more of a neutral substance chromatographically indistinguishable from benzamide (R_f 0.75).

DISCUSSION

The ability of plant tissue to condense applied indoleacetic acid with aspartic acid or ammonia is not restricted to indoleacetic acid, for it is shown by a variety of carboxylic acids. Thus indolepropionic acid, indolebutyric acid and even benzoic acid are con-

FIG. 3. Radioactive metabolites of C^{14}-carboxyl-labeled benzoic acid from pea epicotyls. Radioautographs of chromatograms of tissue extracts: 1) ether extract at pH 7.0; 2) ether extract at pH 2.6; 3) *n*-butyl alcohol extract at pH 2.6. (St.) is a marker of radioactive benzoic acid. The mobile, neutral substance (R_f 0.75 in 1 and 2) is chromatographically indistinguishable from benzamide. The slow moving acidic substance (R_f 0.14 in 2) is benzylaspartic acid.

verted, in greater or lesser degree, to the amides and to derivatives of aspartic acid. However indoleformic acid is converted only to a neutral substance, probably its amide, and no indoleformylaspartic acid is found. 2,4-D is outstanding in that it is conjugated to a very limited extent and up to 95 % of the 2,4-D taken up can be recovered unchanged.

Previous workers have shown that complexes of unknown composition are produced in the metabolism of 2,4-D by the plant. Weintraub et al (11) found that 2,4-D underwent several transformations in dormant plant tissue during a period of several months. Jaworski and Butts (7) recovered two major derivatives other than 2,4-D in the 80 % alcohol extract of treated bean stems. They considered that one of the unidentified products might be a glycoside containing 2,4-D as the aglycon. It is, however, hazardous to attempt a direct comparison of the results of these earlier studies with those reported in the present paper because of the great differences in experimental techniques such as the method of application and duration of the incubation period.

In the present paper, the metabolism of 2,4-D is compared to that of the other acids. Relative to these acids 2,4-D is hardly metabolized at all. In contrast, indoleacetic acid is, over a wide range of concentrations, so rapidly degraded that only 20 % of that taken up accumulates in the tissue as recognizable indole compounds (2). At concentrations below about 20 mg/l, practically all of the indoleacetic acid which escapes degradation is conjugated with aspartic acid to form the much less active indoleacetylaspartic acid. In spite of the fact that indoleacetic acid is removed from the ambient solution at least three times as fast as is 2,4-D, the level of free 2,4-D in plant tissue greatly exceeds that of indoleacetic acid (except at very high and toxic levels of applied indoleacetic acid). It is tempting to suggest that this difference is one reason for the potent herbicidal activity of 2,4-D for, being resistant both to degradation and to conjugation, 2,4-D remains in the plant tissues as the free acid is able to exert its physiological action long after growth substances such as indoleacetic acid have been metabolized.

The appearance of indoleacetylaspartic acid in tissues treated with indolebutyric acid is an indication of β-oxidation, a reaction which has been previously demonstrated by Fawcett et al (4) in the phenoxyalkylcarboxylic acids. It seems probable that this β-oxidation of indolebutyric acid involves the intermediate formation of indolebutyryl-CoA and indoleacetyl-CoA. Probably the conjugation of indoleacetic acid with aspartic acid also involves CoA. These observations therefore lend some support to the hypothesis that indoleacetyl-CoA is involved in the metabolism of indoleacetic acid (10, 13).

Summary

1. Pea epicotyls were incubated for 24 hours in solutions of various carboxylic acids. After the incubation period the tissues were extracted and the metabolic products of these acids were identified chromatographically.

2. Indoleformic acid (indole-3-carboxylic acid) was in part converted into a neutral substance which is probably indoleformamide. No indoleformylaspartic acid was found.

3. Indolepropionic acid yielded indolepropionylaspartic acid and a neutral substance chromatographically indistinguishable from indolepropionamide.

4. Indolebutyric acid treated tissues accumulated substances which were presumably indolebutyramide and indolebutyrylaspartic acid. In addition considerable amounts of indoleacetylaspartic acid and traces of indoleacetic acid were found.

5. Benzoic acid was rapidly destroyed but that portion which did accumulate in the tissues was predominantly in the form of benzoylaspartic acid. Benzamide and traces of free benzoic acid were also found.

6. In contrast, very little 2,4-D was destroyed and nearly all of the accumulated acid was in the free form. Traces of an acidic 2,4-D derivative, chromatographically indistinguishable from 2,4-dichlorophenoxyacetylaspartic acid, also accumulated.

7. The metabolism of 2,4-D is compared with the metabolism of indoleacetic acid on the basis of parallel experiments. It is suggested that the persistence of 2,4-D in the tissues, which results not only from its resistance to degradation but also from its resistance to conjugation, is one reason for its efficacy as a herbicide.

The authors gratefully acknowledge the technical assistance of Mr. M. W. H. van Ysselstein. They wish to thank Dr. J. R. Robinson for his advice in all matters pertaining to the use of radioisotopes and for the counting of the barium carbonate samples. They also wish to thank Dr. G. R. Bell who developed the riboflavin method of detecting 2,4-D on chromatograms.

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MOVEMENT OF C^{14}-TAGGED ALPHA-METHOXYPHENYLACETIC ACID OUT OF ROOTS[1]

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Alpha-methoxyphenylacetic acid (MOPA) was reported in 1953 to have marked plant-growth-modifying properties and to be readily translocated by bean and other plants (6). When applied to leaves or stems, this compound was absorbed and translocated both upward and downward within the plant. It moved down into the roots and out of them and was absorbed by adjacent or nearby roots of an untreated plant; then it moved upward in the stem of this plant to partially developed leaves which subsequently became malformed (7). This phenomenon of plant regulators moving out of roots, however, is uncommon (7). MOPA moved from various kinds of bean plants to other bean plants and from other broad-leaved plants to bean, causing malformation and inhibition of new growth. There was no evidence at that time that the acid moved from corn to any other plant.

In studying the translocatability of a compound, it is, of course, necessary to identify the compound by either biological or chemical means after it has been moved through the plant. In a previous study (5) the downward transport and exudation of MOPA were proved by applying approximately 150 μgm of C^{14}-carboxyl-tagged MOPA to the stems of several bean plants. These plants were then grown with their roots immersed in aerated tap water. Three days later the water was found to contain radioactivity. A sample of the water was evaporated and the residue partitioned on paper. This residue was identified as MOPA. If any radioactive metabolites or degradation products were exuded by the roots, these were not detectable. These earlier results indicate that some of the methoxy acid was absorbed by the stems, translocated to the roots and exuded without detectable chemical change.

The present investigation is concerned with the exudation of MOPA as affected by its absorption and translocation, the amount of MOPA applied and environmental conditions.

[1] Received April 18, 1957.

MATERIALS AND METHODS

Carboxyl-tagged alpha-methoxyphenylacetic acid was prepared on a micro scale from benzaldehyde and sodium cyanide-C^{14} by a modification of the standard procedure (1, 8) involving the successive preparation of mandelonitrile and mandelic acid and the conversion of the latter to MOPA by methylation with dimethyl sulfate.

Young plants of Pinto bean, approximately 5 inches (ca 12 cm) tall, were grown in aerated tap water or nutrient solution for these experiments. The plants, grown in pots containing soil, were removed, and after their roots were washed free of soil, they were placed in beakers containing the aerated tap water or nutrient medium.

Five μgm of C^{14}-tagged MOPA dissolved in water was then spread evenly on the upper surface of each primary leaf by means of a thin glass rod, making 10 μgm per plant. Each beaker contained three plants. After preliminary tests for periods up to 300 to 350 hours, it was arbitrarily decided that further experiments would be terminated after approximately 200 hours, as this was long enough to demonstrate the pattern of exudation. During this period, 20-ml portions of the solution were taken at successive intervals of 24 hours, except for the first 48 hours during which samples were taken more frequently, to determine the concentration of radioactive exudate. These aliquots were then evaporated in metal planchets and tested for radioactivity. Solutions in the beakers were always readjusted to their original volume and, whenever necessary, correction for self-absorption was made.

RESULTS

The presence of MOPA in the tap water surrounding the roots was first detected about 5 hours after application of 5 μgm of the acid to each leaf. The amount in the water surrounding the roots (the amount exuded less the amount reabsorbed by the plant through its roots and that adsorbed on the glass)

increased, reached a maximum, and then decreased (fig 1). This general pattern was observed in experiments carried out with plants subjected to a variety of environmental conditions. In experiments conducted during the following fall and winter, however, the maximum concentration of exudate in water surrounding the roots was more (50 %) than that in water surrounding the roots of comparable plants grown during the summer (fig 2).

Plants grown with their roots in nutrient solution exuded and reabsorbed MOPA in a pattern similar to those grown with their roots in tap water. There was, however, an apparent depression of the rate of exudation when the roots were maintained in the nutrient. This apparent depression was found to be due to the self-absorption of radioactivity by nutrient salts precipitated in evaporation of the aliquots. The self-absorption was found to be approximately 85 %. The correction for self-absorption showed the amount of MOPA exuded when the roots were in nutrient solution to be the same (within 10 %) as that exuded by roots in tap water.

In these tests, some of the plants were grown with their roots in tap water to which the required macronutrients, including calcium nitrate, potassium dihydrogen phosphate, and magnesium sulfate, had been added. Other plants were grown with their roots in tap water alone for comparison.

The amount of MOPA exuded from the roots was proportional to the amount applied to the leaves over the range of dosages used. Two and one-half μgm of MOPA was applied to each leaf in one group of plants, 5 μgm to each leaf in another group, and 10 μgm to each leaf in a third group. The average ratio of amounts of MOPA exuded by the roots in the different groups of plants was 1:2:4, the same as the ratio of the amounts applied to the leaves. These ratios remained alike throughout the experiment.

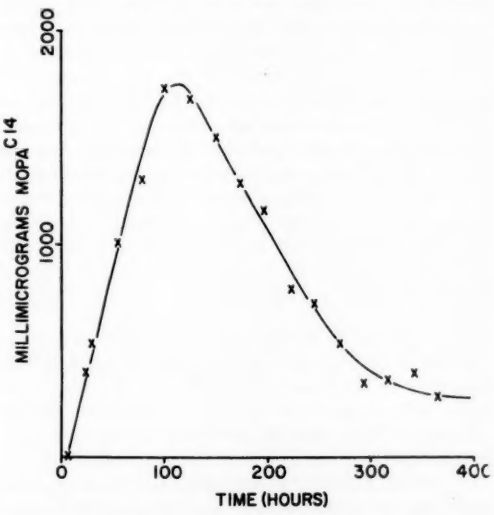

FIG. 1. Average detectable amounts of MOPA C^{14} exudate that occurred in tap water surrounding bean roots.

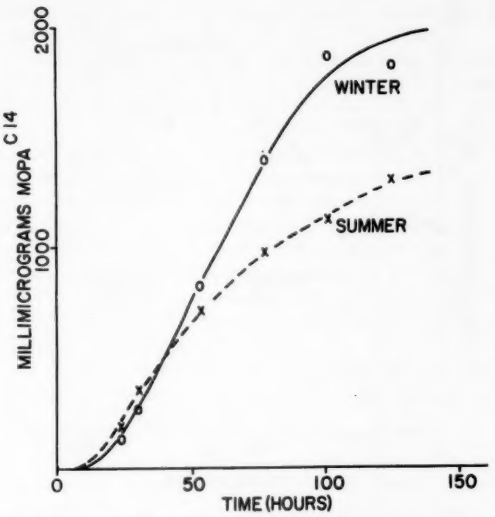

FIG. 2. Average amounts of detectable MOPA C^{14} exudate in tap water surrounding bean roots used in experiments during the summer compared with the average amounts in tap water surrounding roots in experiments during the fall and winter.

Exudation of MOPA was retarded by removal of the lower half of the root system. Pruned roots began to exude detectable amounts of the acid 4 to 24 hours later than did plants with comparable intact root systems. During the early part of the experiment, however, the concentration of MOPA in tap water surrounding pruned roots increased at a somewhat lower rate but eventually reached a higher level than it did in water surrounding the unpruned roots (fig 3). After the concentration of MOPA in water around the pruned roots reached a maximum, it decreased at a rate comparable to that in the tap water containing unpruned ones.

The amount of radioactive MOPA in water around the roots was not affected by continuously subjecting the roots to water containing stable MOPA (30 μgm in 150 ml of water), beginning 48 hours prior to application of radioactive MOPA to the leaves. In these tests, comparable plants with untreated roots were used for comparison. The results were adjusted to account for adsorption of the stable in place of the radioactive MOPA on the glass container.

Exudation of MOPA was greatly reduced by subjecting the roots to a lowered oxygen supply in the water surrounding them. Nitrogen instead of air was bubbled through tap water surrounding the roots to subject the roots to a reduced oxygen supply. Groups of comparable plants with their roots in aerated tap water were used as controls.

Plants subjected to the lowered oxygen supply became somewhat wilted within 1 day and subsequently

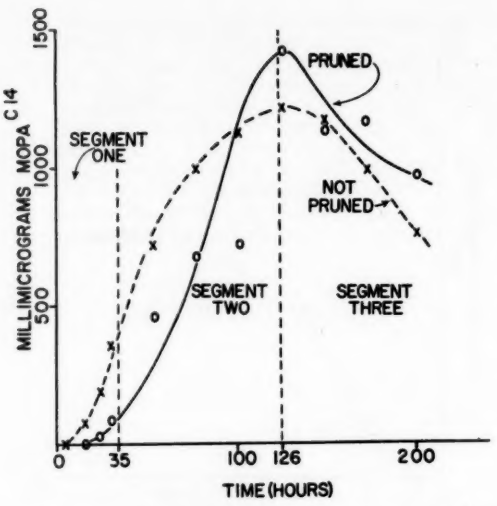

FIG. 3. Average amounts of detectable MOPA C^{14} exudate in tap water surrounding pruned bean roots compared with the average amounts in tap water surrounding roots that were not pruned.

grew very little. Although plants in water of lowered oxygen content exuded a detectable amount of MOPA at about the same time as did plants in aerated water, the radioactive exudate was greatly reduced by the lack of oxygen. During the latter part of the experiment roots in water with reduced oxygen exuded only about 20 % as much acid as did plants with their roots in aerated water.

DISCUSSION

The amount of MOPA absorbed by the leaves, the amount translocated and exuded by the roots, the amount adsorbed on the glass container, and also the amount of the exuded MOPA reabsorbed by the plant through its roots are factors which account in part, at least, for variation observed in the amount of the acid in the water.

It is obvious that during the period in which the amount of MOPA in the water increased, exudation exceeded the rate of reabsorption. As absorption of MOPA by the leaves and its translocation to the roots decreased with time, reabsorption of the acid by the roots exceeded exudation. This apparently accounts for the marked decrease in the amount of MOPA detected in the water during the latter part of the experiments.

A possible explanation for parts of the decrease that occurred in the amount of MOPA in the water during the latter part of the experiment is that bacteria or other microorganisms metabolized some of the MOPA in the water and loss of radioactive carbon dioxide from the water resulted. Both the aerated tap water and the nutrient solution remained clear. Notwithstanding this, some of the MOPA may have been metabolized by the organisms and lost as $C^{14}O_2$.

In considering the effect of the amount of the acid supplied to the leaves on the amount present in the water around the roots, one has to take into account the rate of absorption by the leaves, translocation through the stems into the roots and the rate of exudation from the roots. Since the amount of MOPA in the water was directly proportional to the amount applied to the leaves, it seems apparent that none of the processes mentioned were functioning at a maximum rate, even when 10 μgm was applied to each leaf.

Factors which influenced the amount of MOPA in water around the pruned roots were as follows: first, the effect of reduced root surface area due to removal of the immature portion of the root; secondly, the effect of initiation of new roots due to pruning along with mobilization of plant constituents associated with this production of new roots; and finally, a decrease in the amount of MOPA absorbed and translocated to the pruned roots with the resultant depression in the amount of MOPA in the water surrounding them.

Removal of the lower part of the roots involved loss of the less mature portions including many root tips. This, along with the fact that the total surface area of the roots was greatly reduced, temporarily resulted in a marked reduction in the amount of acid in the water. Thus, the water surrounding the pruned roots contained only 13 % as much MOPA after 35 hours as did that surrounding unpruned ones as shown in segment 1 (fig 3). The lack of a sudden increase in the amount of MOPA in the water immediately after pruning (up to 30 to 40 hours) indicates that a detectable amount of MOPA was not exuded from the injured surfaces.

Considering now the second segment of the curve (fig 3), the marked increase in the amount of MOPA present in the water around the pruned roots may have been partly due to the rapid increase in lateral root development with its resultant increase in root surface. Utilization of carbohydrate, nitrogenous and other plant constituents required in the production of new roots was notably accelerated as the result of the removal of the lower half of the roots. When exogenous compounds are applied to plants, they, together with carbohydrates, are translocated downward (2, 3, 4, 9, 10). This accelerated mobilization of carbohydrates required for root production probably increased the rate at which MOPA was translocated downward and thus resulted in a somewhat greater rate of MOPA exudation than occurred in the unpruned roots.

In segment 3 of the curve (fig 3), the reduced amount of MOPA in water around the roots may have been due to decreased absorption by the leaves and decreased transport of the acid to the roots, thereby allowing reabsorption by the roots to be the controlling factor.

Turning now to a consideration of plants with roots exposed to stable MOPA and leaves treated with radioactive MOPA, the roots absorbed the non-radioactive acid from the solution around the roots. This absorption was obvious from the fact that the stable

form of the acid caused the plant to develop detectable morphological responses in the young leaves even before the radioactive MOPA was applied. The absorption and upward transport of stable MOPA did not interfere detectably with absorption and downward transport of radioactive MOPA from the leaves. Furthermore, the movement of the radioactive MOPA out of the roots and its partial reabsorption by the roots were not detectably affected by the presence of the stable acid.

It should be mentioned, however, that radioactive MOPA in the water surrounding the roots reached a greater level when the roots were placed in water containing stable MOPA before the radioactive form of the acid was applied to the leaves. This increase was apparently due to adsorption of the stable MOPA on the surface of the glass container before the radioactive MOPA was exuded by the roots. This reduced the amount of radioactive MOPA adsorbed on the glass surface and thus caused an increase in the amount detected in the water.

An amount of radioactive MOPA approximately equal to that in water around roots of plants in previous experiments (1.5 μgm per 150 ml) was added to water in a beaker. The amount adsorbed to the glass was about 28 % of the amount added. This percentage of the amount of MOPA detected in the water surrounding the roots was, therefore, subtracted to make the results comparable.

Assay of ground parts from plants subjected to a reduced oxygen supply revealed that relatively little radioactive MOPA was translocated from the leaves to the roots of these plants (80 % less radioactivity in roots exposed to a lowered oxygen supply than in roots with an adequate oxygen supply). This probably accounted for the fact that relatively little MOPA exudate was detected in the water surrounding the oxygen-deficient roots.

Summary

1. C^{14}-labeled MOPA was applied to primary leaves of young Pinto bean plants growing in aerated tap water or nutrient solution so that exudation and reabsorption of the acid by the root could be studied.

2. The amount of MOPA exudate present in the water surrounding the roots (the amount exuded from the roots less the amount reabsorbed by the plant through its roots and that adsorbed on the glass) increased, reached a maximum, and then decreased within a period of approximately 200 hours. This general pattern was observed in experiments carried out with plants subjected to a variety of environmental conditions.

3. Plants grown with their roots in nutrient solution exuded and reabsorbed MOPA in a pattern similar to those grown with their roots in tap water.

4. The amount of MOPA exuded from the roots was proportional to the amount applied to the leaves over the range of dosages used.

5. Exudation of MOPA was retarded by the removal of the lower half of the root system. Pruned roots began to exude detectable amounts of the acid 4 to 24 hours later than did plants with comparable intact root systems. The concentration of MOPA in tap water surrounding pruned roots increased at a somewhat lower rate and eventually reached a higher level than in water surrounding the unpruned roots.

6. The amount of radioactive MOPA exuded into water around the roots was not affected by continuously subjecting the roots to water containing stable MOPA beginning 48 hours prior to application of the radioactive MOPA to the leaves.

7. Exudation of MOPA was greatly reduced by subjecting the roots to a lowered oxygen supply in the water surrounding them.

The authors wish to express their appreciation for the technical assistance of Dr. Wilkins Reeve, Chemistry Department of the University of Maryland.

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THE STANDARDIZATION OF POA ANNUA AS AN INDICATOR OF SMOG CONCENTRATIONS. I. EFFECTS OF TEMPERATURE, PHOTOPERIOD, AND LIGHT INTENSITY DURING GROWTH OF THE TEST-PLANTS[1]

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Of the plants commonly found in Los Angeles County whose responses to smog[2] have been under extensive observation, *Poa annua* (annual bluegrass) was selected as one worthy of intensive study. It was early reported as one of the most sensitive of the plants observed in the field (11). Because it has a regular habit of growth, can be easily and quickly grown in small containers, and because the band of damage caused by smog is measurable, *Poa annua* lends itself to quantitative work. It was therefore thought that it might be standardized to serve as a biological indicator of smog concentrations. A plant indicator has a unique value, since it measures smog in terms of the damage potential to the living cell. It also gives a visible record of the effects of smog conditions at the time and place at which it was exposed.

The technique of filtering from the atmosphere its plant damaging components by means of activated carbon filters was devised in 1950, in a project sponsored jointly by the California Institute of Technology, the University of California, and the Los Angeles County Air Pollution Control District (4). Comparison could then be made between plants grown in air containing smog, and those grown in the same conditions in pure air. It became evident to many observers that certain portions of the mesophyll tissues were acted upon selectively by smog, and that damage was usually found in areas of rather young tissue.

Noble (12) described the pattern of damage found in many species, including *Poa annua*, with special attention to the location of damaged areas as a function of the maturation of the leaf. Bobrov (3) made an anatomical study of *Poa annua*, in which she showed by photomicrographs the relation of susceptible portions of the blade to cells in the young-mature stage. Older cells were shown to be more heavily suberized.

Loftfield (9) in 1921 had shown that alfalfa was susceptible to sulfur dioxide only during those hours in which the stomata were open. Middleton, Kendrick and Schwalm (11) described the microscopic appearance of damaged areas on the leaves of a number of broad-leaved crop plants, noting that damage occurred especially in the mesophyll in the region of the stomates. Bobrov (2, 3), using sections of fresh tissue, showed that the bands of damage (fig 1) found on the blades of *Poa annua* correspond in position to the region of young mature cells whose stomates have newly become functional.

FIG. 1. *Poa annua* plant 4 wks old, after a 1-day exposure to a heavy smog. The light-colored band on each blade, in which the chlorophyll was destroyed, resulted.

Stomatal behavior had been studied also in a series of investigations begun in 1949, sponsored jointly by the California Institute of Technology and the Los Angeles County Air Pollution Control District. In the course of these, Koritz and Went (8) found that the stomata of tomato plants closed as soon as fumigation began, and after repeated treatments closed permanently in many cases. Transpiration rates dropped after each fumigation in every case, even in plants whose stomata were closed at the start of fumigation. It was concluded that although conditions favoring the opening of stomata are also those in which plants are most susceptible to smog damage,

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[2] The term smog in this article will refer to the air pollution existing in Los Angeles County during 1955 to 1956, chiefly the partial oxidation products of unsaturated hydrocarbons.

there was no conclusive evidence that the extent of damage was determined by the degree of opening of the stomata. The fact that further injury is inflicted by smog when the stomata are closed, would indicate that penetration can occur during closure.

It was then found by Hull and Went (6) that the stomata of oats closed tightly when fumigation began, as did those of tomato plants, but those of endive remained wide open during a 2.5-hour fumigation period. Transpiration dropped during fumigation in both species. Some effects of different pretreatment were observed; with 5 crop plants, smog damage was more severe if they had been grown in natural daylight rather than in artificial light, except in the case of alfalfa; and more severe if they had been well supplied with water than if watered only enough to prevent wilting.

Studies were made on the smog response of the Pinto bean by Middleton, Kendrick and Darley. They concluded that it was a suitable indicator plant for the presence of oxidants, inasmuch as damage from ozone could be readily distinguished from damage due to oxidants; and in 1954 used the plant in a study of the relation of plant damage to oxidant level as measured by the release of iodine from potassium iodide. Plants were exposed at 5 stations in the Los Angeles Basin; the percentage of leaf area injured was estimated and given a rating called the plant injury index, or quantitative estimate of damage. Little significant correlation was found between the plant injury index and the oxidant maxima, or between the plant injury index and the oxidant 24-hour mean. The percent of plants damaged was termed the qualitative estimate of injury, and this estimate did show a significant correlation with both oxidant 24-hour mean and oxidant maximum values. The studies were believed to support the thesis that the potassium iodide method of measuring oxidant was measuring, not the phytotoxicant itself, but a concomitant condition.

A complete review of air pollution as affecting plants may be found in an article, with a bibliography, by Donald F. Adams (1); also in section 9, by Thomas and Hendricks, of "The Air Pollution Handbook" (10).

In 1952 test boxes were designed by Noble in which plants could be exposed to smog in an airstream of controlled rate. Species which might be good indicators of smog concentrations were studied by him as to their endurance in test box conditions. Since differences in environmental factors were known to affect the sensitivity of plants to smog, plants were grown in controlled conditions in the Earhart Laboratory, then set out in the test boxes. Spinach, endive, alfalfa, sugar beet, oats, and annual bluegrass were studied in this manner throughout the year.

Of these, the annual bluegrass proved hardiest. It had the added advantage of being so common locally that it may easily be found in the vicinity of any test box, when field observations are desired as a check on test box findings.

Test boxes were put in operation in September 1954, at 12 locations over the county, usually at farms or nurseries. Since then, a set of 10 *Poa annua* plants has been placed in the test chamber each day in each location.

Petunia "Rosy Morn" has also been placed in the boxes, for comparison. As it is a plant of quite different characteristics, it seems unlikely that both species would become insensitive or ultra-sensitive due to the same climatic factors.

Daily, the damage to *Poa annua* has been measured and on this basis the responses to smog have been judged light, medium, or severe. Results are studied in connection with chemical methods of determining smog concentrations, with meteorological data, and with damage to plants growing in the fields nearby. A complete description of the test boxes and their operation has been prepared, together with data showing the correlation of test plant injury with field injury (13).

Methods

Poa annua was grown in various combinations of day and night temperature, photoperiod, and light intensity in the Earhart Laboratory at intervals of approximately 6 weeks throughout the year. At the age of 4 weeks the plants were taken out and exposed to smog in standard test boxes. Damage to sets of plants from the different conditions was assessed and compared. The plants were also measured to obtain growth rates. In some cases fresh and dry weights were taken, or weights were taken of similar sets. Plants of different ages were also compared in the same manner.

The Earhart Laboratory has been fully described elsewhere (15).

Temperatures employed were the following:

Day temp., °C	Night temp., °C
17	11 (cold)
20	14 (cool)
23	17 (moderate)
26	20 (warm)
30	24 (hot)
23	14
26	14
26	17

These were combined with natural daylight and artificial light. Several light intensities were produced by shading plants in daylight with copper screen, and by using artificial light of 700 ft-c and of 1000 ft-c. Most of the above temperatures were combined with 8-, 12-, and 16-hour photoperiods, and two light intensities were tried with each of these combinations.

At the same time, sets of plants were tested which had been grown in a greenhouse built by the Los Angeles County Air Pollution Control District for the regular production of plants for the air pollution test box. This greenhouse, referred to as the "A. P. Greenhouse," is located at the Los Angeles State and County Arboretum in Arcadia. It has a high degree of con-

trol of environmental factors. In winter the temperature is 17° C at night, and rises to a maximum of 27° C at midday. The maximum temperature lasts about 2 hours, the average for the day being 26° C. The heat sum per 12-hour day is from 286 to 290 degree hours, averaging 288. This is the same as in a day of warm conditions at the Earhart Laboratory, the main difference being that in the A. P. Greenhouse, the temperature rises gradually to a peak, as it does outdoors, and gradually declines, instead of holding a constant level throughout the day, as in the Earhart Laboratory. In summer, in the A. P. Greenhouse, the slightly higher range of 19° at night to 30° C in the daytime is maintained, the sum for a 12-hour day being about 300 degree-hours, and for the night, 230 degree-hours.

The temperatures and humidities in the A. P. Greenhouse are recorded on a hygrothermograph so that conditions are known for periods during which each experimental group is being grown. Light intensity is very close to that of outdoors. Supplementary lighting is given in winter to complete a 16-hour photoperiod, and is also supplied on cloudy days. Light intensity is reduced as needed by window-screening placed across the roof. Humidity is not allowed to fall below 60 %. Smog is removed by passing the air through activated carbon filters.

The smog to which the plants were exposed was that of the atmosphere at Arcadia. The test boxes change air 4 times per minute. They have control chambers in which air is forced through a filter of activated charcoal. Thus, in cases of doubt, smog damage may readily be distinguished from injury due to other causes. The plant compartments of the test-boxes have glass walls, and the boxes may be oriented so that the plants are in sunlight all day, or so that they are in the sun morning and afternoon, but are shaded by the opaque door at noon. In these experiments, plants were placed in the test-box before 10:00 A.M. and were removed at 4:00 P.M.

An attempt was made to expose them, so far as possible, on days of comparable smog attacks. During each of the exposures, there was mild sunshine, the temperature was between 60° and 65° F, and reduced visibility and eye-watering were observed in the early afternoon.

After exposure to smog, the grasses were placed in the A. P. Greenhouse for three days to await full development of damage, after which time the extent of the injury was estimated.

For estimating damage, the following method was used: the length of each of the 3 uppermost mature leaves was measured. (The youngest visible blade has been observed to incur damage only after it is at least half as long as the next-older blade. When shorter than this, it is not considered as one of the 3 uppermost mature blades.) The length of the band of damage was also measured and expressed as percent of the blade length. The damage to the 3 blades was then averaged, giving a score for the plant; finally the scores for all the plants in a group, or statistical unit, were averaged, giving the final score in terms of percent of damage per blade per plant. Analyses of variance made on a number of runs indicated that 10 plants are sufficient to give statistically significant values. However, not less than 3 cups with 10 plants in each were used as a unit in these studies.

The depth, or severity, of the smog damage was also recorded, in the following terms: light, if there were only flecks or streaks of white; moderate, if there was no green color left within the band of damage except for a line along the midrib; severe, if all chlorophyll within the band of damage was destroyed.

Usually, when a large proportion of the plants was severely damaged, the bands of damage were long, so that good correlation was found between the two manifestations of injury. But this was not invariably the case. The more consistent results were obtained by measuring the length of the bands of damage. It was felt that until the significance of the severity of damage is better understood, it was best to simply record it, without attempting to integrate it with the measurements.

Usually, the demarcation at the end of a band of severe damage is clear; but sometimes the chlorosis shades out gradually above and below the severely damaged area. In such case, the measurement was made to include, besides the severely damaged part, approximately half of the lightly damaged portion.

For planting, plastic cups 3 inches in diameter and 6 inches deep, with holes for drainage, were used as containers. The planting medium was vermiculite, and seeds were sown on the surface. Nutrients were supplied by watering to field capacity once a day with a modified Hoagland's solution. During the exposure to smog, the cups stood in trays containing half-strength nutrient solution to a depth of half an inch.

Preliminary tests were carried out to determine satisfactory planting media, proper planting depth, and number of plants to a cup. These will not be reported in detail, but data are available upon request.

The stock of seed came from a strain of *Poa annua* which had been standardized by the U. S. Soil Conservation Service. Although *Poa annua* is not apomictic as are most members of the genus, this strain had been grown for many years in an isolated field plot at the U. S. Soil Conservation Service nursery formerly at San Fernando, and is as nearly homozygous as could be obtained.

Plants grown under different conditions varied in form to such an extent that it was sometimes difficult to be sure which plants were the larger, without weighing them. Since this meant uprooting them it could not always be done, so the weights of duplicate sets of 60 to 90 plants were taken.

For obtaining growth rates of living plants, a figure was obtained by multiplying the length and width of the longest blade and the number of blades. This gave good correlation with dry weight. It was less satisfactory after lateral shoots developed. Simply the length of the longest blade times its width was a good

index, at the age at which the plants were used for testing. To get the average for a group of 90 plants in units of 10 per container, the length and width of the longest blades were measured on any 3 plants taken at random from each container. When there were as few as 30 plants in a group, all were measured.

Stomatal opening was studied by means of xylene infiltration, porometer readings, and the ultrapak microscope. When xylene was used, it was applied to four blades of at least 10 plants in each condition. Thirty plants were used when available.

To determine whether the wave-like spread of the xylene was due entirely to infiltration through the stomata, or whether the xylene might be spreading through the interior of the leaf also, grass blades were coated with silicone over part of their length, and xylene was applied at a little distance from the edge of the silicone. There was at times some spreading of the xylene beyond the coating; but as it was relatively slow and limited in extent, it was not thought to be a source of error.

Humidity was adequate for stomatal opening in both the Earhart Laboratory and the test boxes.

Results

Germination and Early Development: Germination occurred in about 7 days, except in the cold conditions, where 18 to 22 days were required. Light shading produced a slightly higher percentage germination when the photoperiod was 16 hours and light intensity was high. In the highest temperatures (day 30° C, night 24° C) there was some loss of very young seedlings.

In the optimal or near-optimal conditions of a warm day and cool night, with a 16-hour photoperiod and natural daylight reaching a maximum of 5000 ft-c, the two-leaf stage was reached about one week from emergence. The growth rate became more rapid at about the development of the 3rd leaf. The 5-leaf stage was reached 3 weeks after emergence. Lateral shoots then began to develop. Flowering began in from 5 to 7 weeks. At the onset of flowering, the uppermost blade, which heretofore had been longer than the one preceding it, developed to only about half the expected length and showed little evidence of stomatal opening. For this reason and because of their size, plants at this age were no longer desirable as test plants. Four weeks was the preferred age.

With the same light intensity and photoperiod, young plants developed most rapidly in the warm temperatures (day 26° C, night 20° C), and in warm days with cool nights (day 26° C, night 17° C). Growth was progressively slower in the moderate, cool, and cold conditions, and was also slower in the hot ones (day 30° C, night 24° C). Relative to the length of the blade, plants in cool conditions had broad blades and long internodes. The opposite was true of plants in hot conditions (fig 2).

Sensitivity to smog developed first in the plants in

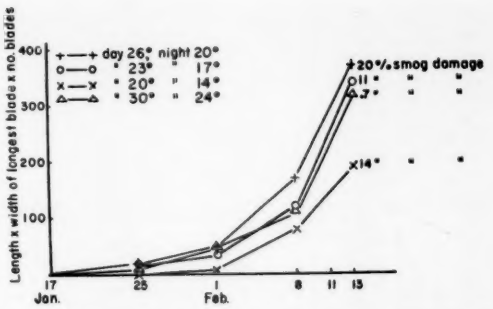

Fig. 2. Growth of *POA annua* in 4 combinations of day and night temp. The photoperiod was 16 hrs, and the light intensity up to 4200 ft-c at noon. Plants in cool temperatures have slower growth rate than those in hot ones, but suffered more damage from smog.

the hot conditions, which incurred damage as soon as the first blade expanded. This sensitivity tended to be lost by the time they had 3 or 4 leaves. They did not become immune to smog, but suffered less damage than did plants in other temperatures. Sensitivity to smog was absent in plants in cool temperatures (day 20° C, night 14° C) at the 2-leaf stage, but increased as they grew older, up to the age of 6 weeks, when it declined. Young plants in warm and moderate conditions showed sensitivities intermediate between those of hot and those of cool ones. In the cold (day 17° C, night 11° C), plants did not become sensitive to smog until they were beginning to flower.

Interaction of Temperature and Light Intensity: Growth in 4 temperature combinations is shown in figure 2. It is typical of the growth of Poa in these temperatures throughout most of the year. In January, however, plants grown in cool and in moderate conditions made the best growth, while in August those in hot conditions did so. There appeared to be an interaction of light intensity and temperature such that plants growing in high temperatures made better growth in high light intensities, while those in low temperatures grew better in lower light intensities.

This was most clearly seen when artificial light (700 and 1000 ft-c) was combined with cool, warm, and moderate temperatures. Figure 3 shows the results of this experiment. The same response had previously been found in tomatoes (16).

In the warm day, cool night combination (day 26° C, night 17° C) the effect of light intensity was minimized; the plants weighed the same in both light intensities. There was a relative difference in wet and dry weight, fresh weight being greater at 700 ft-c and dry weight at 1000 ft-c. This combination was also optimal for growth.

Temperature had a decided effect on susceptibility to smog. Plants grown in hot conditions (day 30° C, night 24° C), received much less smog damage than did those from any other condition (fig 2). This was true whether the hot-condition plants were the largest,

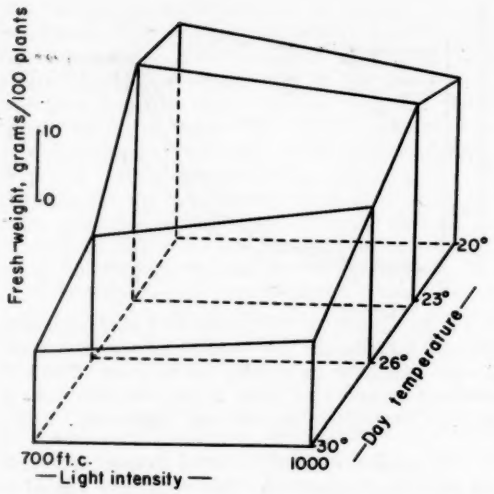

FIG. 3. Effect of various temperature and light intensity combinations on the growth of *Poa annua*. Artificial light and a 16-hr photoperiod was used. Night temp was 6° lower than day temp. In cool and moderate temperatures, growth is better with the lower light intensity; in warm and hot temperatures, with the higher intensity.

as when they were grown in high light intensity, or whether they were relatively small, as when grown in lower light intensity. In some of the trials they sustained no damage at all.

Small differences in degree of injury were always found among plants grown in cool, moderate, and warm conditions. The greatest damage from smog was incurred by plants grown in warm conditions (day 26° C, night 20° C) or in a warm day with a somewhat cooler night (day 26° C, night 17° C). This latter combination seemed somewhat better for both growth and smog sensitivity. With a still cooler night (day 26° C, night 14° C) growth was not as good.

OTHER EFFECTS OF LIGHT INTENSITY; OPTIMAL INTENSITY: When light reached the plant from all sides, as when plants were grown one to a cup, the effect of high natural light intensity was to induce early formation of lateral shoots. In an experiment in which 1, 2, 5, 10, and 20 plants per cup were grown in the A. P. Greenhouse in May, the lateral shoot development was in inverse proportion to the number of plants per cup. By 4 weeks, the area filled by the grass blades was about the same, whether the blades proceeded from one large plant or 5 to 10 smaller ones. Because of this, plants of the age to be tested had no freer air flow among their blades, nor less shading of one blade by another, when planted one to a cup than when planted 10 to a cup. With 20 to a cup, the stems were thin and weak, and the plants appeared to have suffered from crowding.

In the Earhart Laboratory, the optimal intensity did not appear to be exceeded. The largest plants were raised in warm temperatures with natural light, and under these conditions they did best in full sunlight. Plants placed next to them but shaded by one layer of gauze, did not attain the same size. The optimal intensity was then sought in the A. P. Green-

TABLE I

EFFECT OF PHOTOPERIOD ON GROWTH AND SUSCEPTIBILITY TO SMOG OF 4-WEEK-OLD POA ANNUA

DAY TEMP, °C	NIGHT TEMP, °C	PHOTOPERIOD, HRS	LENGTH OF LONGEST BLADE, MM	WIDTH OF LONGEST BLADE, MM	NO. OF BLADES	NO. OF LATERAL SHOOTS	TOTAL DRY WT, GM/100 PLANTS	SMOG-DAMAGE	
								% PER BLADE PER PLANT	PLANTS WITH SEVERE DAMAGE, %
20	14	8 A	45	1.0	4.0	0	0.24	21	100
"	"	12 A	71	3.0	5.0	2	0.83	*	83
"	"	16 A	95	3.0	5.0	2	1.90	17	96
23	17	8 A	60	2.0	4.0	0	0.27	16	96
"	"	12 A	80	2.5	6.0	2	1.34	*	*
"	"	16 A	110	3.5	6.5	3.5	3.80	10	84
26	20	8 A	74	1.5	4.0	0	0.30	15	96
"	"	12 A	60	2.0	4.0	0	0.47	*	*
"	"	16 A	66	2.0	4.0	2	0.87	10	79
30	24	8 A	35	1.0	4.0	0	0.12	4	100
"	"	12 A	*	*	*	*	0.31	*	*
"	"	16 A	52	2.0	5.0	0	0.42	2	24
20	14	8 N	63	2.2	4.5	0	4.1	18	12
"	"	16 N	128	3.3	5.0	0	11.8	29	5
30	24	8 N	14	0.5	2.0	0	0.2	Traces	0
"	"	16 N	*	*	*	*	1.3	1	0

A = artificial light, N = natural light. Measurements are averages of 30 plants; wts are averages of 30 to 60 plants. Damage is termed severe when there is a band in which all chlorophyll is destroyed.

* No data.

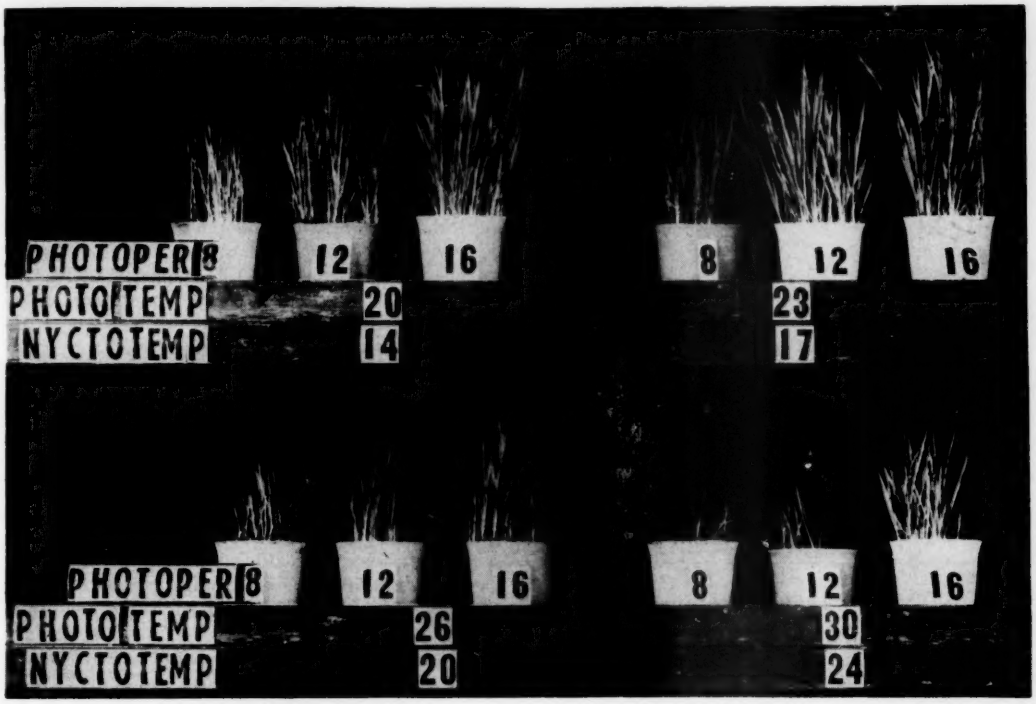

FIG. 4. Five-week-old *Poa annua* grown in 4 temp combinations, each with 3 photoperiods.

house, where light intensities are almost as high as outdoors. Here plants did better unshaded, both as to germination and growth, up to the month of May. In May, plants shaded by window-screening weighed more. There was a higher root-shoot ratio, but the blades were also larger; the figure for length × width × number of blades being 862 for unshaded plants, 2065 for shaded ones. According to data from the nearest U. S. Weather Bureau Station, the unshaded plants received in May a total of 14,000 langleys, or a daily average of 485 lys with a maximum of 748 lys. In April, when the unshaded plants had been slightly larger, they had received 12,677 lys, or a daily average of 408 lys with a maximum of 645 lys. This probably is optimal. Data from the weather station correlated well with light meter readings taken several times a day at the greenhouse.

EFFECT OF PHOTOPERIOD: A 16-hour photoperiod produced plants that were larger in every respect than did a 12-hour period, and this in turn, than an 8-hour period. This was true in each of the 4 temperature conditions. In the hot conditions, a photoperiod of at least 12 hours was necessary for good survival as there were always many dead seedlings in the 8-hour photoperiod (fig 4). In cool conditions, the 8-hour photoperiod produced plants that were small but quite healthy.

The effect on smog sensitivity may been seen in table I. Damage was consistently greater in the 8-hour photoperiod.

When plants were grown in cool conditions and a short photoperiod, they were remarkable for the consistency of the smog damage. An analysis of variance was made, and bore out the impression that the variation in amount of damage among the individual plants was small. There was a definite pattern, with damage at the base of the third leaf in every case. The areas of severe injury were clearly demarcated.

Even the plants grown in the hot conditions showed fair sensitivity when in an 8-hour photoperiod and shaded, provided their water-supply was maintained at field capacity. Maintenance of this plentiful water supply did not produce such sensitivity in plants in a 16-hour photoperiod nor in the unshaded plants in the 8-hour photoperiod and the same temperature.

EFFECT OF CHANGING PLANTS TO DIFFERENT TEMPERATURES: When plants were changed to different temperatures, the immediate result for the first 3 days was an increased growth rate, compared to the growth rate of plants which remained in the same conditions. Plants transferred from hot to cool temperatures gained in sensitivity; those transferred from cool to hot ones, lost it. After 3 days, the plants were exposed to smog, and the damage measured. The difference between each transferred group and its control group was not significant except in one case. This was the transfer from warm conditions, which produced plants of the highest sensitivity, to hot condi-

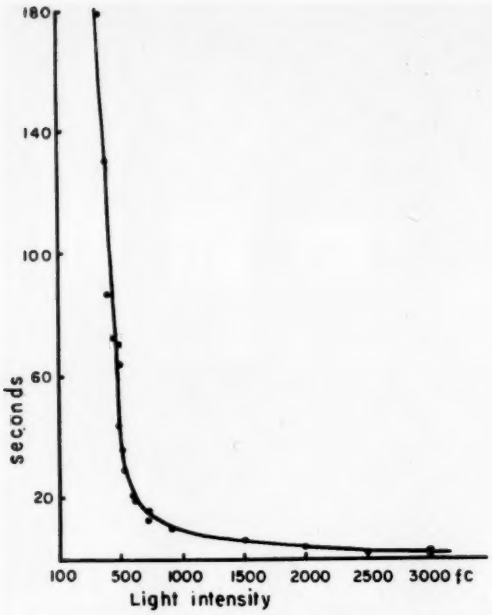

FIG. 5 A. Correlation of light intensity with degree of stomatal opening, as indicated by porometer readings, in plants grown in cool temperatures.

tions, which produced plants of the lowest sensitivity; and the reverse transfer, from hot to warm conditions.

It was not determined how long this increased growth rate would have persisted, as the plants were removed to the test-boxes after 3 days. It was observed, however, that plants which were senescent in high temperatures, became rejuvenated in the cool temperatures, and flowered again. The largest plants ever obtained by any treatment were sown in cool temperatures, given 5 cold nights one week after germination, then gradually brought through moderate temperatures to warm ones. This parallels natural conditions in a Mediterranean climate, where *Poa annua* is an autumn germinating annual.

EFFECT OF TEMPERATURE AND LIGHT ON STOMATAL OPENING: When evidence of stomatal opening was sought by applying xylene to the blades of 3- to 4-week-old plants, the color change due to infiltration appeared in bands strikingly similar in pattern to bands caused by smog damage. This had been previously noticed by Bobrov and Vasek. Bobrov (3) had correlated the damage patterns with the location of young-mature tissue, in which the stomata are most active.

Figure 5 A is based on figures obtained by estimating the areas of infiltration in terms of percent of the blade, as is done in scoring smog damage, and obtaining the average percent per blade of a large group of plants. The plants in this instance were tested at noon on a sunny day, in the Earhart Laboratory. Distinct differences in the stomatal opening of plants growing in different temperatures may be seen. Plants in the A. P. Greenhouse at the same hour on a number of days of comparable light, showed stomatal opening similar to that of the warm conditions (day 26° C, night 20° C) in the Earhart Laboratory, or a little greater.

A correlation with smog damage may be observed, in that plants from warm conditions, which have stomata open over more of the blade-length than do any other group, also incur the greatest smog damage; while hot-condition plants incur the least.

The timing of stomatal opening also differed among the 3 groups. Maximal infiltration began earlier and continued longer in the plants grown in warm conditions, than in cool-condition plants. Plants grown in hot conditions gave evidence of stomatal opening only before 9 A.M. This would be of importance in smog exposure because smog at the test site never reached damaging concentrations so early in the day.

Very young plants, in the 1- to 2-leaf stage, showed infiltration with xylene in only 3 out of 10 plants at most, in all conditions.

An ultrapak microscope and a porometer were used to try to gain an idea of the width of the opening. On days of mild sunshine, in both warm and in cool conditions, wide open stomata were interspersed with partly open ones and with tightly closed ones, in the proportion of 3:5:3. In hot conditions no fully-open stomata were ever observed.

Porometer readings were difficult to obtain in hot conditions. When areas could be found in which the stomates were open, they closed in about half an hour. Frequently by shifting to another part of the same blade, other open ones could be found, but they in turn soon closed. Figure 5 B shows a typical set of porometer readings on plants growing in cool conditions. There was mild sunshine on the day of the readings. Stomatal opening responded sensitively to light intensity. There was a 15-minute lag in response, observed also on other occasions when pass-

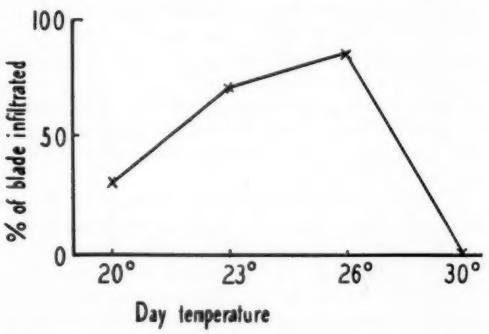

FIG. 5 B. Correlation of temperature with the leaf area over which stomates were open, in natural light at 2 P.M. on a clear day. Xylene was applied to the blades and the infiltrated areas estimated as % of blade. Averages of 10 plants. Night temp were 6° lower than day temp in each case.

ing clouds changed the light intensity. There was a threshold for opening in the morning, at about 400 ft-c.

Experiments in shaded and unshaded test boxes were carried out to see what might be the practical effect of the response to light intensity. In theory, the width of the stomatal opening should not matter, because it would follow from the principles of diffusion of gases through small apertures, that a stomate need not be fully open to permit the maximum diffusion of gases. Hence small changes in light intensity should not affect the amount of smog damage. If however the light intensity fell below the threshold of 400 ft-c, the stomata should be completely closed, and the plant be quite resistant to smog.

This was found to be the case. When the test box was shaded so that the light intensity was only 300 to 400 ft-c, only traces of injury were found on the shaded plants, while the damage to plants in the unshaded box was 15 % per blade per plant (based on 9 containers of plants, 10 plants per container). When the test box was shaded less heavily, permitting light intensities of 900 to 1200 ft-c, damage to plants was within 2 or 3 % of the damage incurred by plants in the full light intensity of 3000 to 4000 ft-c. (This latter experiment was done 4 times with the same results; on a fifth repetition there was a somewhat greater difference between the test plants and controls—18 % damage to the former, 32 % to the latter.)

When plants were changed from cool temperatures where the stomata were open over a large proportion of the blade, to hot conditions where the stomata were nearly all closed, the stomata of the transferred plants did not at once begin to close. This was shown by xylene infiltration with groups of 20 plants on several occasions. Also, in several experiments a plant with a porometer attached to the blade was wheeled from a cool room to a hot one, both of which were sunny. The blade continued to give readings consistent with the curve already begun, and with curves obtained previously in the cool room. Conversely, plants transferred from a hot room to a cool one at the same light intensity, did not give evidence on the same day of opening their stomata over larger areas. Only after one or two days did the stomatal behavior alter until it was characteristic of plants in the temperature condition to which the plants were moved.

Lateral Shoots: Lateral shoots had about the same percent of area damaged as did the central shoots, if they were nearly the same size. When plants were grown whose lateral shoots all reached the same development, and the same as that of the central shoot, the smog damage was of the same proportion on all. Hence it was decided that the laterals were, essentially, repeating the results of their parent shoot and only the parent need be scored.

Correlation of Growth Rate and Smog Sensitivity: No correlation could be found with smog sentivity, of either the growth rate of the plant as a whole, or of the young mature blades. So far as elongation could be detected by placing India ink lines 1 mm apart along the young blades, the blades had ceased to elongate by the time they became sensitive to smog. In the experiments in which growth rates were measured, the plants growing fastest during the three days previous to the smog exposure were not the ones most severely damaged. For example, plants in the 8-hour photoperiod and cool conditions were growing at a much slower rate than those in warm ones, yet damage was equally severe; conversely, when plants in hot conditions were growing fastest of all, they were still the least smog-sensitive group.

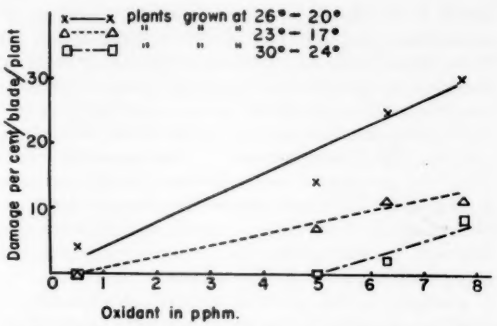

Fig. 6. Correlation of plant damage with oxidant readings at nearest station. Each point represents the damage from 1 exposure to smog (av of 30 plants). During all exposures, there were temperatures of 60° to 65° F, mild sunshine, and a peak of high oxidant readings lasting 2 hrs. All plants were grown in natural daylight with a 16-hr photoperiod.

Correlation with Total Oxidant Levels: Figure 6 shows the correlation between total oxidant in the air, and the severity of damage of *Poa annua*. Each point represents the average damage caused by a single exposure to smog to plants raised in a given condition. The plants were grown in the Earhart Laboratory in natural light and 4 temperature combinations. Total oxidant reading in parts per hundred million were obtained from the nearest station. This was at the California Institute of Technology, where the phenolphthalein method was used. The figures plotted were the maximum for the day; in each case the maximum came in the early afternoon and lasted about 2 hours. Temperatures and light intensities were comparable. In these experiments the relative sensitivity of plants grown under different conditions remained the same; that is, plants grown in 26°–20° C were always more sensitive than those grown in 23°–17° C, and those in turn were more sensitive than those coming from 30°–23° C. This last condition is considered an unsatisfactory one in which to grow test plants.

Discussion

While conditions in which *Poa annua* are grown have a very strong effect on its sensitivity, it seems remarkable, compared to other plants with which we have had experience, for the breadth of the range in

which it is sensitive. If one is content with a differentiation between the effects of light smog and heavy smog, only one condition need cause concern; namely, too great heat. Somewhat greater precision than this, however, can be attained in any one of a number of combinations of temperature and photoperiod. The light-optimum of *Poa annua* is rather high, but in optimal temperatures the plants make good growth and are smog sensitive over a wide range of intensities. The optimal conditions are such that they can be maintained in a relatively simple glass house such as the A. P. Greenhouse.

Petunia, barley, *Atropa belladonna,* and several other smog-sensitive plants which have been grown in controlled conditions have had narrower ranges in which they were sensitive, or in which the sensitivity was quantitatively reliable. Petunia, sensitive when grown in hot conditions, is good for use with *Poa annua.*

An apomictic species of Poa would be more uniform genetically and therefore would have advantages as an experimental plant, but so far no apomictic Poa has been found with the wide temperature-tolerances of *Poa annua,* with the possible exception of a hybrid of *Poa scabrella* and *Poa pratensis* which, with others of the genus, was tested in the Earhart Laboratory (5, 7).

The fact that the small plants from the 8-hour photoperiod and cool temperatures were as sensitive as large ones from optimal growth conditions could be accounted for by the assumption that the mature cells age more slowly than they do in warmer temperatures and with more light. There is some evidence for this, in that the old blades do not become senescent nearly as fast. If, as has been believed, the length of the band of damage in proportion to the whole leaf depends on the length of the band of newly matured tissue produced just previous to exposure, it would seem at first thought that a rapidly growing plant would have a longer band of damage than a slower growing one. However in a plant which is also aging slowly, the cells might remain longer in the stage at which they are vulnerable, and the proportion of damageable tissue be the same as in the larger plants. The length of the band would thus be determined by the balance between maturation and aging. This could explain the lack of correlation of sensitivity with growth rate.

The data afforded by the stomatal studies do not altogether account for the absence of damage to very young plants in cool conditions. Possibly this is due to the compactness of the cellular structure. While the lack of injury to plants grown in hot conditions seems accounted for by the timing of stomatal opening with reference to the onset of smog, it may also be that suberization occurs more rapidly in high temperatures. More work is needed along these lines.

In the curves in which total oxidant was plotted against plant damage, the data plotted were obtained when conditions of exposure (duration of the highest oxidant readings, temperatures, light intensities) were quite comparable. The inference is not that the daily routine tests would show such a correlation; in these, a general correlation has been found (13), but complete correlation is not to be expected; obviously a number of factors could prevent this. It is felt, however, that the correlation shown in figure 6 strengthens the conclusion, drawn also from other data, that in each set of environmental conditions *Poa annua* develops a degree of smog sensitivity characteristic of that set of conditions. Between some sets of conditions, the difference is slight; between others it is quite pronounced. Proper choice of growing conditions must therefore be made, and these conditions scrupulously maintained, in order to use *Poa annua* successfully as an indicator. Furthermore, certain requirements, such as sufficient light intensity, must be provided during the exposure to smog. It is planned to present further data on test box conditions in a later communication.

It is felt that our study supports the thesis that plants, properly handled, constitute excellent test materials for smog conditions. They give a quantitative expression of smog in a visible form which may be preserved; and they give it in terms of its effect on the living organism.

Conclusions

The environmental conditions under which *Poa annua* is grown strongly affect its sensitivity to smog. Temperatures of day 26° C, night 20° C in the Earhart Laboratory, or the equivalent in daily heat sum in the A. P. Greenhouse, combined with a 16-hour photoperiod and natural light, produce plants of satisfactory sensitivity. These conditions have been adopted for the production of test plants for the daily tests carried on by the District.

Temperatures of day 30° C, night 24° C or their equivalent in daily heat sum in the A. P. Greenhouse, and with the above photoperiod and light intensity, produce plants which are resistant to smog and are considered unsatisfactory for testing purposes. Intermediate temperatures produce plants of intermediate sensitivity.

With 8-hour photoperiods and a somewhat lower light intensity, equally sensitive plants may be produced, in combination with any one of several temperatures. The interaction of temperature, light intensity, and photoperiod is such that it is not possible to consider the effect of one without the others.

A partial explanation for the differing sensitivities of plants grown in different conditions is afforded by the evidence that the stomata have a different habit of opening in each condition.

Since transfer of plants from cool to hot temperatures, or vice versa, did not result in appreciable changes in smog sensitivity in less than three days, nor in alteration other than gradual in their habits of stomatal opening, it is felt that a change in environmental conditions during the testing period does not impair the usefulness of the plants for smog testing purposes. This may not hold true if the change is

extreme: hence, supplementary light for dark days, and cooling devices for periods of extreme heat, are recommended.

Small and slowly growing plants may be as sensitive as large ones, under certain conditions. These conditions—cool temperatures, shade, short photoperiod—are such that aging takes place slowly. Hence, a proportionately large amount of tissue may remain in the young-mature, susceptible stage for some time, thus compensating for the slow production of fresh susceptible tissue.

Summary

Poa annua (annual bluegrass) was grown in controlled conditions in the Earhart Laboratory in 8 temperature combinations, natural and artificial light of various intensities, and 3 photoperiods. It was also grown in the A. P. Greenhouse in which the day temperature rose gradually to a maximum lasting about two hours. Growth rates, fresh and dry weights were obtained.

For growth up to the age of 4 weeks, optimal conditions were a day temperature of 26° C, night 17° C, with a 16-hour photoperiod and a high light intensity. There was an interaction of light intensity with temperature, so that plants in high temperatures grew better with high light intensities, on the order of 12,000 ft-c at noon; while plants in low temperatures grew better with light intensities on the order of 3,000 ft-c at noon. With artificial light, this interaction with temperature was also observed, using intensities of 700 and 1000 ft-c. Plants in the warm and moderate temperatures were less affected by variations in light intensity, within those limits.

Eight-, 12-, and 16-hour photoperiods produced progressively better growth. Survival was poor in the 8-hour photoperiod with high temperatures.

Flowering was earlier and more abundant in the cool temperatures with short photoperiod.

Smog sensitivity was tested by exposing the plants to atmospheric smog for one day in test-boxes in which airflow and humidity was regulated. Three days after the smog exposure, the length of the 3 youngest leaves and of the bands of damage on them was measured and damage expressed as percent per blade per plant. A unit of 3 containers with 10 plants in each was found ample to overcome variations due to genetic or unknown factors.

Sensitivity developed at the age of 10 days from planting, even before the second blade had grown out, in plants in hot conditions; but thereafter diminished. At 4 weeks, damage in heavy smog was less than 5 % per blade per plant. Sensitivity in plants grown in cool conditions did not develop until about the 3-leaf stage, but did not diminish until the plants were more than 6 weeks old. High sensitivity (20 % to 30 % damage per blade per plant from heavy smog) was found in plants grown under several conditions: 1) optimal growth conditions; 2) 8-hour photoperiod in moderate light intensities, and cool, moderate or warm temperatures; and 3) 16-hour photoperiod in the A. P. Greenhouse, with a day temperature reaching a maximum of 27° C and a night temperature of 17° C.

Plants grown in hot conditions gained sensitivity when transferred to cool conditions. Those grown in cool conditions lost sensitvity when changed to hot ones. An appreciable gain or loss in sensitivity to smog required about 3 days.

Smog sensitivity was not correlated with the size of plants, nor with the growth rate. It was correlated to a high degree with the area over which the stomates were wholly or partly open. It was also correlated with oxidant levels, in data obtained when duration of maximal oxidant readings and climatic conditions were comparable.

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PHYSIOLOGY OF THE CELL SURFACE OF NEUROSPORA ASCOSPORES. IV. THE FUNCTIONS OF SURFACE BINDING SITES[1,2]

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The previous paper in this series (6) has disclosed that the surface of Neurospora ascospores can serve as a reservoir of cations which can enter the cell after adsorption. For example, Ag^{+}, UO_2^{++}, Cu^{++} and other cations are removed from solution by dormant live ascospores and by killed ones as well. Furthermore, the kinetics of uptake and the fact that these materials can be eluted readily suggest that they are localized on the cell surface. If the dormancy of such "coated" ascospores is broken they fail to germinate due to the presence of the toxic cations on their surface. This effect has been demonstrated in the case of organic bases like Polymyxin-B (5) as well as for the substances mentioned above. These observations suggest, therefore, that the cell surface is the source of cations which are excluded from vital centers in the spore until germination is induced.

However, the fact that such absorbed ions can penetrate does not establish that this route is obligatory. It is also possible that the cations must be eluted before they enter and that the adsorption sites merely concentrate them; or, alternate means of penetration may exist which are independent of preliminary surface localization.

That surface adsorption of cations is widespread is demonstrated by its occurrence in bacteria (7, 9), erythrocytes (1), fungi (10, 13) and higher plants (8, 17). Therefore, an investigation of the relation of adsorption to penetration was undertaken in order to define the role of this ubiquitous phenomenon.

MATERIALS AND METHODS

Ascospores of *Neurospora tetrasperma* were obtained, stored and prepared for use by the methods described previously (12). The latter paper also describes the techniques used in the heat-activation as well as the germination of the spores. The concentration of these cells was determined by the use of a Klett colorimeter with a blue filter (Klett # 42) whereby a reading of 180 corresponded to a concentration of 1 mg (dry wt) per ml of spore suspension.

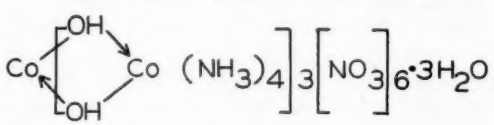
"Coated" ascospores were prepared by mixing equal volumes of suspensions containing 1 mg per ml of ascospores and 2×10^{-2} M of the unbuffered "coating" material. This mixture was incubated at 20° C on a reciprocal shaking machine for 24 hours whereupon the supernatant fluid was decanted after centrifugation and the ascospores washed in 4 changes of water. The "coated" ascospores were resuspended in water and stored at 4° C until used.

The measurement of oxygen uptake was performed by the standard manometric procedures outlined in Umbreit et al (15). Unless otherwise noted, the Warburg vessels contained 0.5 ml of the spore suspension, 0.5 ml of a solution of the cation to be tested, or an equivalent volume of water, and 0.2 ml of KOH in the inset as an absorbent for carbon dioxide. All respirometric experiments were carried out at 26° C at a shaking rate of 120 oscillations per minute. The dry weight of the spore suspensions used was obtained by keeping aliquots in an oven at 105° C overnight.

Ag^{110} was determined by means of an end-window Geiger tube and scaler. As before, aluminum pans containing 0.2 ml of the solution were used and enough counts were recorded to assure less than 2 % counting error.

Hexol nitrate (see list of abbreviations) was prepared according to the method of Werner (16), as modified by Sutherland (14). The cobalt hexammine salts were generously provided by Prof. Robert W. Parry of the Department of Chemistry, University of Michigan. List of abbreviations:

1. PCB: phenylmercuribenzoic acid.
2. Hexol nitrate: hexa-ethylenediamino-hexoltetracobaltic nitrate whose chemical formula is



This and the subsequent description of cobalt coordination compounds is used as provided in Sidgwick (11).

3. Cobalt hexammine: hexammine cobalt (III) chloride whose formula is $Co(NH_3)_6Cl_3$.

[1] Received April 25, 1957.
[2] This work was made possible by grants from the Rackham Graduate School, University of Michigan, and from the American Cancer Society.

Results

As a result of the disruption of dormancy in ascospores of Neurospora a 20-fold enhancement of oxygen uptake is observed (4). Such metabolic activity is a sensitive indicator of the effect of toxic materials upon the cells (12) and serves to indicate the point where such inhibition is first manifest. With these possibilities in mind, the effect of adsorbed cations upon the oxygen uptake of ascospores was studied.

To accomplish this, a suspension of ascospores that had been "coated" with UO_2^{++} was activated and its oxygen uptake compared with that of a similar but untreated suspension of spores that served as a control. In addition, the oxygen uptakes of untreated spores suspended in 1×10^{-3} M $UO_2(NO_3)_2$ immediately and 105 minutes after activation were also studied. The results provided in figure 1 demonstrate that no effect of the UO_2^{++} is obvious until 100 minutes of incubation after activation. At this time the oxygen uptake of both "coated" spores and those suspended in a UO^{++} solution after activation is inhibited although the effect of the latter treatment is much greater. However, in no case did germination occur.

It is worth mention that the effect of the UO_2^{++} that was tipped at 105 minutes was almost immediate although the amount of inhibition was somewhat lower than in the case where the cation was included in the spore suspension from the start.

This experiment was repeated with spores that were treated with Ag^+ in the same manner as above except that the effect of tipping at 105 minutes was not studied. As in the case of spores treated with

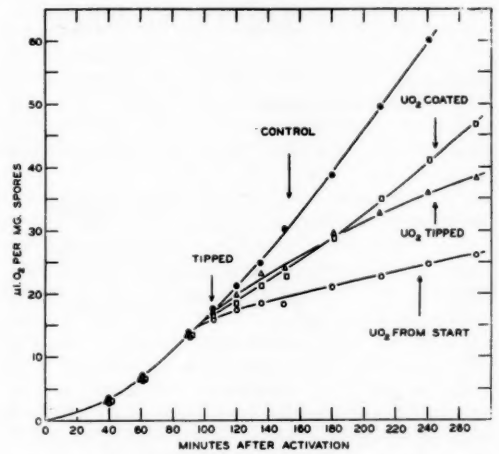


Fig. 1. Effect of UO_2^{++}-"coating" upon the oxygen uptake of activated ascospores of *N. tetrasperma*. "Coated" ascospores were treated with 1×10^{-3} M UO_2^{++} for 24 hrs after which they were washed free of the residual metal as described in the text. Enough of the salt was added at 105 minutes after activation to give the same final concentration in the tipped samples. Each point is the average of the readings of 3 vessels.

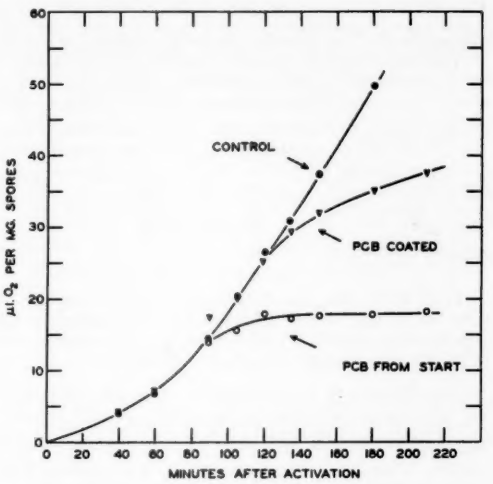


Fig. 2. Effect of PCB-"coating" upon the oxygen uptake of activated ascospores of *N. tetrasperma*. "Coated" ascospores were treated with 1×10^{-3} M PCB for 24 hours after which they were washed free of the residual metal as described in the text. Each point is the average of the readings of 2 vessels.

UO_2^{++} those treated with Ag^+ were affected only after 100 minutes.

In order to test the effect of an organic cation upon this system, phenylmercuribenzoic acid (PCB) was used in the same way as were the inorganic substances. Again, as in the latter instances no effect of PCB was demonstrable until incubation had proceeded beyond 90 to 100 minutes after activation (fig 2). However, in this case, the "coated" cells were affected at least 20 minutes after those that had been incubated continuously in the poison although the former cells still did not germinate. An experiment was also performed in which the oxygen uptake of dormant PCB-"coated" cells was compared with that of untreated ones and it was found in both cases that the Q_{O_2} (μl oxygen per mg dry weight per 60 minutes) was about 0.5.

It had been reported previously (13), that certain strongly basic substances like Polymyxin-B, protamine and complex salts of cobalt served to prevent the adsorption of methylene blue on ascospores of *N. tetrasperma*. This observation provided a means whereby the role of adsorbing sites in the penetration of cations might be tested. For example, if adsorption of cations on surface sites is a necessary prelude to their penetration, then the blocking of such sites by the materials listed above should prevent this process. Alternatively, if adsorption is not required, then the entrance of cations should proceed unimpeded by such treatment.

Accordingly, experiments were designed to test whether cobalt hexammine chloride and hexol nitrate prevented the surface-binding of Ag^+, as well as that

TABLE I

UPTAKE OF Ag^{110} *by* ASCOSPORES OF *N. tetrasperma* AFTER TREATMENT FOR 24 HOURS WITH COMPLEX COBALT SALTS AT A CONCENTRATION OF 1×10^{-2} M

TREATMENT	CPM AFTER 30 MIN	UPTAKE IN CPM	PERCENTAGE REMOVAL
Hexol nitrate	81	108	57
Cobalt hexammine	76	113	60
Control	23	166	88
Starting solution	189	...	..

Final concentration of ascospores was 1.0 mg per ml and that of Ag^{110} was 2 μgm per ml. Incubation in the mineral was continued for 30 minutes. The figures provided are the average of triplicate counts.

of methylene blue, to ascospores. Dormant cells were "coated" with each of these substances and, after being washed free of the complex salts, aliquots containing 1 mg per ml of the spore suspensions were mixed with an equal volume of a solution containing 4 μgm per ml (3.7×10^{-5} M) of Ag^{110} and incubated on a shaking machine at 20° C for 5 hours. At this time the cells were precipitated by centrifugation and aliquots of the supernatant fluid were pipetted onto planchets and their radioactivity determined as described before. Exactly similar treatment was accorded a set of untreated ascospores which served as the control in this experiment. The results provided in table I show that the adsorption of Ag^+ by dormant ascospores is markedly interfered with by previous treatment with cobalt hexammine chloride and hexol nitrate.

It was now possible to examine the effect of blocking adsorbing sites upon the uptake of Ag^+ by ascospores after activation. With this in mind, aliquots of Ag^+-, hexol nitrate-, and cobalt hexammine-coated ascospores were made up to 16 mg per ml and activated as was a suspension of untreated spores at the same concentration. Thereupon, aliquots of these spore suspensions were diluted to 8.0 and 4.0 mg per ml and all were incubated at 30° C for 1 hour after activation. At this time, an equivalent volume of a solution containing 4 μgm per ml Ag^{110} was added to each of the flasks containing the spore suspensions described above and incubation was resumed for a total of 2.5 hours after activation. The ascospores were then removed by centrifugation and aliquots of the supernatant were plated out for counting of residual Ag^{110}. Since the time of incubation had been large enough to assure the protrusion of a germ tube, the percentage germination was determined after the spores had been killed by the addition of formaldehyde. The results provided in table II lead to the conclusion that Ag^{110} is not excluded from the cell by the presence of any of the surface "coatings" used; in fact, the amount taken up is greater under these circumstances. Moreover, although germination was complete in all the spore suspensions to which water had been added, none occurred in those which had been treated with Ag^{110}.

TABLE II

UPTAKE OF Ag^{110} BY VARIOUS CONCENTRATIONS OF ACTIVATED ASCOSPORES TREATED WITH HEXOL NITRATE, COBALT HEXAMMINE, OR Ag^+

SPORE CONC MG/ML	CONTROLS	SPORES "COATED" WITH		
		HEXOL NITRATE	COBALT HEXAMMINE	Ag^+
		Uptake of Ag^{110} in cpm		
2.0	34	30	29	87
4.0	54	84	77	134
8.0	82	117	128	140

Incubation of the spores in the metal was performed as described in the text. The final concentration of Ag^{110} was 2 μgm per ml and the initial counts in this solution totaled 176 cpm.

Another way of testing the effect of "coating" materials is to determine whether such treatment alters the rate at which Ag^+ inhibits the oxygen uptake of activated ascospores. Accordingly, activated spore suspensions that had previously been coated with hexol nitrate were added to Warburg vessels as before and enough Ag^+ tipped 105 minutes after activation to give a final concentration of 5×10^{-3} M. As can be seen in figure 3, the oxygen uptake of "coated" cells is inhibited as rapidly as that of controls so that the penetration of Ag^+ does not seem to be affected by such treatment. Identical results were obtained when cobalt hexamine was used instead of hexol nitrate.

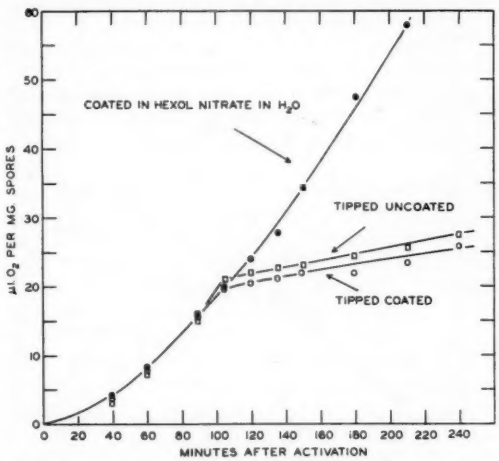


FIG 3. Effect of "coating" with hexol nitrate upon the time when Ag^+ affects the oxygen uptake of activated ascospores of *N. tetrasperma*. Final concentration of Ag^+ after tipping 105 minutes after activation was 1×10^{-2} M. Points represent the average of readings in duplicate vessels. Germination: control, "coated" with hexol nitrate—91%; tipped, "uncoated"—0%; tipped, "coated"—0%.

Discussion and Conclusions

The above results suggested that prior adsorption to sites on the cell surface did not result in faster penetration to the interior of the cell or to the sites that are sensitive to heavy metals. In fact, in the data summarized in figures 1 and 2, the inhibition by Ag^{+}, UO_2^{++}, and PCB of cells "coated" with these materials took longer to materialize than in the cases where the cells were incubated continuously in these substances. This was so despite the fact that the former cells had been exposed to the cations at least 24 hours previous to the latter. These data are supported by the results of the experiments which demonstrated that the blocking of adsorbing sites with hexol nitrate or with cobalt hexammine did not reduce the amount of Ag^{+} taken up by the cells after activation. Furthermore, the effect of these substances upon the oxygen uptake of germinating ascospores was not delayed by such blocking. Therefore, these results lead to the conclusion that surface binding of cations is not a prerequisite to penetration, at least in ascospores of *N. tetrasperma*.

Abundant confirmation of the fact that ascospores undergo a drastic change in permeability as a result of activation is furnished by these experiments. For example, figures 1 and 2 demonstrate that the activated cells become permeable to cations only after they have been incubated for 105 minutes under these conditions. This is also supported by previous data (5) showing that cells "coated" with Polymyxin-B, a basic polypeptide, has the same effect as do the cations studied above. It might be argued that these data could be accounted for by assuming that slow diffusion into the activated cells is possible throughout the course of incubation and that it takes about 100 minutes for toxic amounts to accumulate. That this is not very likely is suggested by the fact that the first indication of the effect of each of the cations tested occurs at the same time. Furthermore, when UO_2^{++} was tipped 105 minutes after activation, instead of being added to the spores from the start (fig 1), there was no lag in the expression of the poison's effect, suggesting again that a barrier to penetration is removed or modified to permit the entrance of cations at this time.

According to Rothstein and Hayes (10), the binding sites on the surface of the yeast cell are associated with less than 2 % of the total cellular cations. These sites must, therefore, involve only a small fraction of the cellular structure. This calculation was made for Neurospora ascospores on the basis of previous work (12) in which was reported the amount of K^{+}, Na^{+}, Mg^{++} and Ca^{++} to be found in these cells. Because only a negligible amount of cations other than those are present the total concentration is approximately 0.785 micromoles per mg dry weight of ascospores. It should be noted that this figure is several-fold higher than in the case of yeast but this is understandable, perhaps, in view of the fact that the ascospores are a type of resting cell and store relatively large amounts of food materials. Using the data provided in the previous paper (6) surface bound Ag^{+}, UO_2^{++}, Cu^{++}, Th^{++++} and Al^{+++} account for 9.8, 11.3, 5.5, 2.7 or 3.8 %, respectively, of the total cations in the cell. In all the cases studied, therefore, a larger proportion of cations is bound to the surface of Neurospora than to that of yeast.

Whether this is the case for other resting cells is still not known but the concentration of trace elements on the cell surface from the external environment would provide such cells with a ready supply of such materials when needed. From the practical standpoint, the presence of relatively small amounts of a toxic cation could result in the inhibition of germination. The data in tables I and II suggest that "coating" the cell results in the enhancement of uptake during germination so that it is possible to consider such treatment as acting as an adjuvant would in the application of fungicides. It is possible, then, that one way in which cobalt hexammine and hexol nitrate act is by neutralizing charges on the surface of the cell.

If the assumption is made that the cations bound to the surface of the dormant ascospore are arranged in a single layer then the proportion of the surface that is occupied can be calculated. However, before this could be done it was necessary to calculate the surface area of the ascospore and, to this end, 100 measurements of its axes were made with the results averaging 31.7 and 14.9 μ. Examination shows that the ascospores have approximately the shape of prolate spheroids, that is, of ellipsoids which are generated by the rotation of ellipses

$$x = a\cos\theta;\ y = b\sin\theta \qquad (0 < b \leqslant a)$$

about the x-axis. By somewhat extended but elementary computations,[3] it can be shown that the area A(a, b) of such an ellipsoid is given by the formula

$$(1)\quad A(a,b) = 4ab\pi \int_0^1 \sqrt{1 - c^2u^2}\, du \quad \text{(Edwards, 1932)},$$

where $c^2 = 1 - \frac{b^2}{a^2}$. It is readily seen that the value of the integral in the last expression lies between $\pi/4$ and 1. In other words, $A(a, b) = 4ab\pi\lambda$; where λ depends on the ratio b/a, but lies in every case between 1 and $\pi/4$; and in any case $A(a, b) \leqslant 4ab\pi$. As judged from the ratio of the axes of the ascospores (a/b), the extreme values of the integral given above are 0.92 and 0.82, while the average is 0.85. Therefore, the average surface area of the ascospores was computed by substituting 0.85 for the integral in (*1*) and the absolute maximum was obtained by setting this equal to 1, with the following results:

Maximum Area 3.0×10^{11} sq. Å
Average Area 2.5×10^{11} sq. Å

By reference to the tables of ionic radii presented by Ephraim (3) it was now possible to compare the area available on the cell surface to that occupied by

[3] The authors are indebted to Prof. George Piranian of the Department of Mathematics, University of Michigan, for the derivation provided below.

TABLE III

THE SURFACE AREA OCCUPIED BY CERTAIN IONS ASSUMING THAT THEY ARE ARRANGED IN A SINGLE LAYER ON THE SURFACE OF THE NEUROSPORA ASCOSPORE

ION	ACTUAL IONIC RADIUS IN Å	NO. OF MOLEC. PER SPORE *	AREA OCCUPIED PER SPORE IN SQ. Å
Al^{+++}	0.40	1.0×10^{11}	2.01×10^{11}
Th^{++++}	1.08	7.2×10^{10}	1.05×10^{12}
Ag^{+}	0.74	2.6×10^{11}	1.82×10^{12}

* Data taken from Lowry, Sussman, and von Böventer (1957).

a single layer of ions used in these experiments. For the purposes of this calculation, it is assumed that the ions are spherical. A consideration of table III reveals that many more molecules are absorbed to the surface than there is space to accommodate them, if the assumption is made that they occur in a single layer. The discrepancy is 4-fold in the case of Th^{++++} and 7-fold for Ag^{+}, using the value obtained for the average area of the spore surface, suggesting that these ions must be arranged in a 3-dimensional pattern on the ascospore surface. Even if the figure for the maximum possible area is used there is still a large discrepancy between the amount of space available on the spore and that required for ions arranged in a single layer. The possibility that the adsorbed materials are arranged in depth is supported by recent work (Lowry and Sussman, unpublished) describing the lamellar structure of the cell wall of Neurospora ascospores.

To summarize it seems clear from the data presented in this paper and in the others in this series that cations adsorbed to the cell surface can enter when germination commences. Therefore, the adsorbing sites might serve as a source of trace minerals for the cell, especially since these are usually multivalent and are strongly bound by the surface. This is in agreement with the idea of some workers (8) that it is more reasonable to consider the concentration of adsorbed minerals on the root surface to be the amount available to the plant rather than that of a dilute nutrient solution.

SUMMARY

The prior adsorption of Ag^{+}, UO_2^{++}, and phenylmercuribenzoate (PCB) to sites on the cell surface did not result in faster penetration to the interior, as judged by their effect upon the respiration of germinating ascospores of *Neurospora tetrasperma*. Moreover, the blocking of adsorbing sites by treatment with hexol nitrate or cobalt hexammine chloride did not result in decreased uptake of Ag^{+} or in a delay in the time when the respiratory effects of Ag^{+}, UO_2^{++}, and PCB were manifested. These results lead to the conclusion that adsorption to the surface of the cell is not a necessary prelude to penetration. Cations bound to the surface account for about 3 to 11 % of the total cations of the cell. Calculations of the area of the cell disclose that adsorbed cations are probably arranged in depth on the cell surface rather than in a single layer.

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CARBON DIOXIDE FIXATION INTO OXALACETATE IN HIGHER PLANTS[1,2]

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In 1938, Wood and Werkman (27) proposed that certain metabolic phenomena in bacteria could be explained in terms of the occurrence of a CO_2-fixing reaction. They suggested that CO_2 is added to pyruvate to form oxalacetate (OAA), as shown in equation (*1*). Since that time considerable information has been accumulated about the detailed mechanism

$$CO_2 + \text{pyruvate} \longrightarrow \text{OAA} \qquad (1)$$

of this "Wood-Werkman reaction." It was recognized early that simple addition of CO_2 to pyruvate occurred with such an unfavorable free energy change that some device for supplying chemical energy was essential if the reaction was to play a quantitatively important role in intermediary metabolism (7). Three different CO_2-fixing processes whereby this end can be achieved have been described to date. These are: a) the malic enzyme (or Ochoa) reaction, b) the phosphoenolpyruvate carboxykinase (or pepcarboxykinase) reaction, and c) the phosphoenolpyruvate carboxylase (or pepcarboxylase) reaction. All of these reactions involve the addition of CO_2 to a C-3 unit, to form a C-4 dicarboxylic acid; and all three reactions must be combined with some other reaction to give the net result shown in equation (*1*).

The focus of current interest has been partially on the detailed mechanism of these different enzyme reactions, and partially on their physiological interrelationships. Although the "malic" enzyme had been shown to be widely distributed (24), only limited information was available about the distribution of the other enzymes. The work described in the present paper was concerned mainly with the problems of assaying pepcarboxylase and pepcarboxykinase, and determining their reaction characteristics and distribution in higher plants. The reaction catalyzed by pepcarboxykinase, first described by Bandurski and Greiner (3, 4), is shown in equation (*2*). The pepcarboxykinase reaction was first clearly defined by Utter and Kurahashi (22, 23), who showed that the enzyme from bird liver catalyzed the reaction shown in equation (*3*).

$$\text{PEP} + CO_2 \longrightarrow \text{OAA} + P_i \qquad (2)$$

$$\text{PEP} + CO_2 + \text{GDP (or IDP)} \rightleftharpoons \text{OAA} + \text{GTP (or ITP)} \qquad (3)$$

(PEP, phosphoenolpyruvate; GDP, guanosine diphosphate; IDP, inosine diphosphate; GTP, guanosine triphosphate; ITP, inosine triphosphate; P_i, inorganic phosphate.)

The present work has shown that the pepcarboxykinase of higher plants uses ADP rather than GDP.

MATERIALS AND METHODS

ENZYME PREPARATIONS: Most of the plant materials were purchased from local groceries. Exceptions were lupine, pea seed, wheat germ and corn. Lupine seeds, supplied by Dr. Eric E. Conn, were germinated as described elsewhere (10). At 5 to 8 days the cotyledons were removed, washed and treated as described below. Pea seed acetone powder was furnished by Dr. Helen Stafford. Spinach leaf acetone powder was donated by Dr. R. H. Nieman. Wheat germ S-50 was supplied by General Mills. Corn seeds (Yellow Dent, Variety WF 9 XM 14), a gift from Dr. Richard H. Hageman, were germinated according to his directions in glass casserole trays on acid-washed sand, with 10^{-4} M $CaCl_2$ added to the casserole dish till the solution just covered the sand. The seeds were put on paper towels placed on top of the sand. The casserole dishes were covered with parafilm paper and placed in the dark at room temperature. After 3 days the parafilm paper was removed, and after one more day the seedlings were collected and washed.

In general, the enzyme preparations used were made by blending the washed plant material in a Waring blendor at moderate speeds with enough 0.001 M phosphate buffer of pH 7.4 to give a smooth homogenate. The mixture was strained through two layers of cheesecloth and centrifuged at 0° C in a Servall refrigerated centrifuge at $500 \times g$ for 5 minutes. The sediment was discarded. Ammonium sulfate was added to the supernatant with constant stirring until the solution was three-fourths saturated. After 10 to 15 minutes, the suspension was centrifuged at 0° C for 10 to 15 minutes at $18{,}000 \times g$. The precipitate obtained was suspended in cold, distilled water and clarified by centrifugation. The clear supernatant was dialyzed for 3 to 4 hours against several changes of distilled water. The dialysate was clarified by centrifugation and the clear protein solution was lyophilized. The lyophilized powder was the source of the enzyme extract. All operations were carried out in the cold at 4 to 8° C.

FRACTIONATION OF PARTICULATES AND CYTOPLASM: Spinach particulates and cytoplasm for the experiments described in table VIII, were prepared as follows. Spinach leaves were homogenized in 0.35 M NaCl as described by Arnon et al (1, 2). All operations were carried out at 0 to 8° C. The large particles and debris were removed by straining through cheesecloth and centrifuging at $500 \times g$ for one min-

[1] Received May 9, 1957.

[2] This investigation has been aided by grants from the American Cancer Society and from the Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

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ute. The supernatant solution was then centrifuged for 30 minutes at 18,000 × g. The supernatant solution obtained from this second centrifugation was the source of the cytoplasmic protein, which was precipitated by ammonium sulfate and lyophilized after salt removal as described in the general procedure. The sediment was washed once by resuspending in 0.35 M NaCl and centrifuging for 20 minutes at 18,000 × g. The precipitate was suspended in cold distilled water and lyophilized. An extract of the powder is referred to as "total particulates." To obtain "intact chloroplasts" and "small particulates," the procedure was the same as that used for preparing total particulates, except that the first centrifugation was carried out at 300 × g, and the suspension was then centrifuged at 1200 × g for 10 minutes, to give a preparation consisting mainly of intact chloroplasts. By final centrifugation for 30 minutes at 18,000 × g, a sediment of smaller particles was obtained. Washing and lyophilization was carried out as for the total particulate fractions, and protein solutions were finally made from the dry powders for the experiments given in table VIII. The "washed chloroplasts" and "washed small particulates" were prepared in the same way except that each fraction was washed three times by suspension and recentrifugation from 0.35 M NaCl before the lyophilization procedure.

The separation of cauliflower mitochondria was carried out as described by Laties (13). The mitochondrial sediment and the supernatant solution from the first high speed sedimentation were treated in an analogous manner to the spinach fractions. That is, the mitochondria were suspended in water and lyophilized. From the dry powder, the soluble protein was extracted to give the solution used in the experiments. The protein from the supernatant was precipitated by addition of $(NH_4)_2SO_4$ to 75 % saturation. The precipitate was redissolved in H_2O and salts were removed by dialysis, as described in the general procedure.

Pea mitochondria were prepared from whole 4-day seedlings ground in 0.2 M sucrose - 0.015 M phosphate of pH 7.0, as described by Price and Thimann (14). The protein of the supernatant from the first high speed centrifugation was recovered by the same procedure as that described for the cauliflower preparations. A protein extract was also prepared from the mitochondria by the same procedure as described for cauliflower.

Reagents: The sodium salts of adenosine diphosphate (ADP), adenosine triphosphate (ATP), guanosine triphosphate (GTP) and inosine diphosphate (IDP) were obtained from Pabst. Crystalline ATP free of other non-adenine nucleotides was used in later experiments and found to give exactly the same results as the amorphous ATP salts. The nucleotides were neutralized with NaOH prior to use. Inosine triphosphate (ITP) was the barium salt from Sigma and was prepared for use as described by Tchen and Vennesland (19) except that NaOH was used for neutralization. Phosphoenolpyruvate (PEP) was obtained from two sources. The Ag-Ba double salt of PEP, a gift from Dr. John Graves, was converted to the neutral Na salt (19). The PEP-tricyclohexylamine salt was purchased from the California Foundation for Biochemical Research. Both preparations of PEP gave similar results in the system employed. Collidine was purchased from Eastman Company. OAA was prepared from the sodium salt of the diethyl ester (Practical) by the procedure of Heidelberger (9). The OAA was neutralized with NaOH just prior to use. DPNH was used as the salt of tris (hydroxymethyl) amino methane, and was a gift from Dr. F. A. Loewus. Protein was determined by the optical method of Warburg and Christian (11, 26).

Procedures and Results

The Exchange Reaction: The pepcarboxykinase reaction was assayed by determination of the exchange of $C^{14}O_2$ into OAA in the presence of ATP (25). The mechanism of this "exchange" is understood as a cycling process, i.e, ATP reacts wtih OAA to give PEP, CO_2 and ADP. The PEP and ADP may then react back with labeled CO_2 to form carboxy-labeled OAA. The reaction has the advantage of high sensitivity as attested by positive results obtained when the reaction products couldn't be detected by chemical analysis.

The experimental procedure involved incubation of the extract to be tested with OAA and $C^{14}O_2$,[4] in the presence and absence of ATP (25). The reaction was stopped by addition of 2 ml of 50 % citric acid to the 10 ml of reaction mixture. The $C^{14}O_2$ was flushed out. Traces of $C^{14}O_2$ were washed out with a stream of unlabelled CO_2, the flushing was repeated, and a sample of the last remaining CO_2 was always collected for radioactivity determination, to ensure that the removal of $C^{14}O_2$ was virtually complete. If the radioactivity determination of the control showed that the $C^{14}O_2$ of the medium had not been entirely removed, the experiment was discarded. After removal of the CO_2 of the medium, the OAA was decarboxylated by addition of 5 ml of aniline citrate reagent. The CO_2 formed was collected in 4 ml of CO_2-free, 1 *N* NaOH. The CO_2 content of an aliquot of this solution was determined manometrically. Then another aliquot (usually 2 ml) of the solution was diluted to 20 ml with CO_2-free water, and sufficient 0.5 M Na_2CO_3 was added to make a total of 250 micromoles of carbonate. The carbonate was precipitated by addition of 0.4 ml of 1 M $BaCl_2$. The $BaCO_3$ was collected by centrifugation and washed with methanol. The washed precipitate was plated, dried and counted at infinite thickness in a gas-flow counter. The counts per minute, corrected for background, were corrected by the known dilution factor to give the counts per minute (cpm) of the collected

[4] The term $C^{14}O_2$ is used here to represent all forms of carbon dioxide and carbonate. During the incubation, the label is present mainly as $HC^{14}O_3^-$, but it may be the unhydrated form, $C^{14}O_2$, which actually participates in the reaction.

CO_2, i.e, of the β-carboxyl group of the OAA. About 50 micromoles of $NaHC^{14}O_3$ containing about 2.5 μc of C^{14} were added to each reaction mixture. This carbonate had a thick sample count in our apparatus of 3×10^6 cpm. All data are given as thick sample cpm determined under identical conditions. The ratio of the specific activity of the β-carboxyl group of OAA to the specific activity of the carbonate of the medium at the beginning of the incubation may be obtained by dividing the figures for "exchange" by the thick sample count of the added $NaHC^{14}O_3$ (3×10^6 cpm).

Table I contains representative results obtained for the exchange reaction with extracts from eighteen different sources. The thick sample count of CO_2 collected from OAA incubated in the absence of ATP was about the same in all experiments, ranging from approximately 200 to 400 cpm. When the incubation mixture contained added ATP, there was a much larger amount of exchange observed in more than half of the cases. The ten sources of the first group in table I all gave a response to ATP of sufficient magnitude to be regarded as unquestionable. Only tobacco leaf and lettuce leaf gave clearly negative responses to ATP. The negative and questionable results are not regarded as proof of the absence of the enzyme, but only as inconclusive. Later experiments showed that extracts of pea seedlings definitely catalyzed the ATP-stimulated exchange, so the results with pea seeds, for example, probably reflect the presence of a small amount of enzyme. The reproducibility of the exchange assay was determined in some instances by as many as five duplicate experiments, and considerable variability in the numerical results was observed. However, the difference between the lowest and highest value of duplicate determinations did not exceed 40 % of the highest figure. It is likely, therefore, that most of the results listed as questionable should be regarded as positive, but the authors prefer to leave the question open in view of possible alternative explanations for small positive effects.

TABLE I

DISTRIBUTION OF THE ATP-STIMULATED EXCHANGE REACTION

ENZYME SOURCE	EXCHANGE ($10^{-3} \times$ CPM)	
	No ATP	10 MICROMOLES ATP
Corn seedling	2.3	90
Squash fruit	3.1	83
Cauliflower bud	3.6	71
Lupine cotyledon	2.3	70
Turnip	2.4	42
Mustard leaf	2.8	24
Radish leaf	4.1	14
Cabbage leaf	2.9	13
Cucumber fruit	1.8	11
Wheat germ	2.9	10
Pea seed	3.6	6.3
Parsnip root	2.6	4.2
Egg plant	2.2	4.2
Honeydew melon	2.6	4.0
Parsley leaf	2.9	3.6
Spinach leaf	1.8	3.4
Tobacco leaf	1.9	1.9
Lettuce leaf	2.0	1.7

The test system contained 100 micromoles of OAA, 100 micromoles of $MnCl_2$, 50 micromoles of $NaHC^{14}O_3$ (3.0×10^6 cpm), 500 micromoles of collidine buffer of pH 7.0, and enzyme, in a total volume of 10 ml. Incubation was for 30 minutes at room temperature, which was generally 23° C. Except for the case of pea seed and spinach leaf, the enzyme was prepared by suspending 20 to 30 mg of lyophilized powder per ml of deionized water, and centrifuging down the insoluble residue. One ml of the clear supernatant solution was added to the incubation mixture.

The preparation from pea seed was made by fractionation of an aqueous extract of the acetone powder with ammonium sulfate. The protein which precipitated between 25 and 70 % saturation was dissolved in water and dialyzed overnight against 0.005 M collidine buffer of pH 7.4. In the assay 1.5 ml of solution, corresponding to 2 gm of acetone powder, was used. In the case of spinach, the acetone powder was extracted with 0.002 M collidine of pH 7.0. The extract was dialyzed overnight against 0.01 M collidine of pH 6.8, and clarified by centrifugation. One ml of material, corresponding to 50 mg of acetone powder, was used in the assay.

TABLE II

REQUIREMENTS OF THE "BLANK" EXCHANGE REACTION

REACTION SYSTEM	EXCHANGE ($10^{-3} \times$ CPM)
Complete	24
No ATP	2.8
Complete, enzyme heat-inactivated	2.7
No enzyme	2.6
No enzyme, no Mn^{++}	0.4
No enzyme, no OAA	0.2

The reaction mixture and procedure were the same as that used in the experiments of table I. Mustard leaf was the enzyme source; and 13 mg of protein were used in the test system. When OAA was omitted during the incubation, it was added later as a carrier to provide CO_2 during the decarboxylation.

The data in table I shows that definitely positive results were obtained with four of the eight families tested. The reaction occurs in both monocotyledonous and dicotyledonous plants, and active preparations were obtained from fruits, roots, buds, cotyledons, seeds and leaves.

THE "BLANK" EXCHANGE: The results in table I show a small and relatively constant exchange in the absence of ATP, no matter what the enzyme source. Further experimentation showed this reaction to be largely non-enzymatic, and dependent on added Mn^{++}. Table II shows the experimental evidence for this conclusion. The complete system contained all the components of the assay, including ATP. It is clear that the same small exchange was observed if the enzyme was omitted, or heat-inactivated, or if the ATP was omitted. When Mn^{++} was omitted, however, the exchange decreased to about one tenth of the amount observed in the presence of Mn^{++}. It seems reasonable to assume that the Mn^{++}-dependent exchange is a

measure of the reversibility of the non-enzymatic Mn^{++}-catalyzed decarboxylation of OAA. The justification for this assumption is detailed in the following paragraph.

To assess the significance of the "Blank" exchange, one may calculate from the best available data how much $C^{14}O_2$ exchange might be expected to occur if the decarboxylation was a one-step reaction and the exchange represented a simple reversal of this reaction. For the reaction

$$OAA^{=} + H_2O \longrightarrow \text{pyruvate} + HCO_3^-$$

Burton and Krebs (6) have calculated the free energy change at unit activity and 25° C, $\Delta F° = -6.39$ kilocal. From this value, the equilibrium constant, $K = \frac{(HCO_3^-)\ (\text{pyruvate})}{(OAA)}$ may be calculated.

$\Delta F° = -RT \ln K$ (where R is the gas constant and T is the absolute temperature). $K = 4.9 \times 10^4$. For the reaction

$$OAA^{=} \underset{k'}{\overset{k}{\rightleftharpoons}} \text{pyruvate} + HCO_3^-;$$

$$K = \frac{k}{k'} = 4.9 :: 10^4 \text{ moles/L}$$

If the amount of C^{14} entering OAA is represented by x, then

$$\frac{dt}{dx} = k' (\text{pyruvate})\ (HCO_3^-)\ \frac{C^{14}}{(HCO_3^-)}$$

[Where $\frac{C^{14}}{(HCO_3^-)}$ is the specific activity of the HCO_3^- at any particular time and the parentheses represent concentrations in moles/L]

This gives equation I:

$$\text{I.}\quad dx = k' \cdot C^{14} (\text{pyruvate})\ dt$$

Divide equation I by an equation II:

$$\text{II.}\quad d(\text{pyruvate}) = k(OAA)dt = k[A - (\text{pyruvate})]dt$$

where A is the initial concentration of OAA.

This gives

$$\frac{dx}{d(\text{pyruvate})} = \frac{k'}{k} \cdot C^{14} \cdot \frac{(\text{pyruvate})}{[A - (\text{pyruvate})]}$$

or

$$dx = \frac{C^{14}}{K} \cdot \frac{(\text{pyruvate})}{[A - (\text{pyruvate})]} d(\text{pyruvate})$$

This expression may be integrated to solve for x when any particular fraction of the OAA has been decarboxylated.

For 50 % decarboxylation,

$$\int_0^x dx = \frac{C^{14}}{K} \int_0^{A/2} \frac{(\text{pyruvate})}{[A - (\text{pyruvate})]} d(\text{pyruvate}) + C$$

$$\int_0^{A/2} \frac{(\text{pyruvate})}{[A - (\text{pyruvate})]}$$

$$= \Big|_0^{A/2} [A - (\text{pyruvate})] - A \ln[A - (\text{pyruvate})]$$

$$= A\ (\ln 2 - \tfrac{1}{2})$$

$$C = 0$$

$$x = 0.19 \cdot \frac{C^{14}}{K} \cdot A$$

or

$$\frac{x}{C^{14}} = 0.19 \frac{A}{K}$$

If

$$A = 1 \times 10^{-2}$$

$$\frac{x}{C^{14}} = 3.9 \times 10^{-8}$$

If $C^{14} = 100$, $x = 3.9 \times 10^{-6}$, and is the % of the total C^{14} which will have been introduced into the β-carboxyl group by the time half of the OAA has been decarboxylated. Since the calculation does not take into account the fixed C^{14} which will be lost again by decarboxylation, the above figure is too high. The amount of OAA which was decarboxylated in the blank sample was generally somewhat less than 50 %, in which case the calculated amount of C^{14} fixed will also be high so the statement is justified that *less* than

TABLE III

CHARACTERISTICS OF THE ATP-STIMULATED EXCHANGE REACTION

TIME OF INCUBATION		ENZYME CONC		Mn^{++} CONC		ATP CONC	
TIME	EXCHANGE ($10^{-2} \times$ CPM)	PROTEIN	EXCHANGE ($10^{-2} \times$ CPM)	Mn^{++}	EXCHANGE ($10^{-2} \times$ CPM)	ATP	EXCHANGE ($10^{-2} \times$ CPM)
min		*mg*		*micromoles*		*micromoles*	
5	8	3.9	38	0	0.5	0	3
15	17	7.8	46	5	1.8	5	14
30	28	12.9	170	25	15	10	25
60	72	20.7	390	50	24	25	25
..	..	...	...	100	43	50	30
..	..	...	...	...	..	100	24

Except for the variant under examination, the conditions were the same as described in the legend for table I. Each reaction mixture contained 3.5 mg of protein from turnip. The data are not sufficiently accurate to warrant the conclusion that the departures from linearity, with time and with protein concentration, are real.

TABLE IV

COMPARISON OF Mg^{++} AND Mn^{++}

ENZYME SOURCE	EXCHANGE ($10^{-2} \times$ CPM)		
	NO METAL	Mg^{++}	Mn^{++}
Turnip	2.0	7.6	47
Cauliflower bud	2.2	6.1	68
Squash	4.8	19	63
Lupine cotyledon	1.8	52	69

Conditions as in table I. One hundred micromoles of the chloride salt were added.

3.9×10^{-6} % of the total C^{14} would be introduced into the β-carboxyl group.

The calculated value may now be compared with the value actually observed in the blank. The thick sample count of the CO_2 from the 50 micromoles of OAA left at the end of the reaction was generally about 3×10^2 per minute. The thick sample count of the 50 micromoles of $HC^{14}O_3^-$ present in the medium initially was 3×10^6 counts per minute. The ratio $3 \times 10^2 / 3 \times 10^6 = 1 \times 10^{-4}$, or 1×10^{-2} percent of the C^{14} added was actually observed to be fixed. This is three orders of magnitude greater than the calculated value. Though the free energy data and the experimental data are subject to considerable error, these errors are probably not large enough to account for the large discrepancy between the experimental result and the calculated result. This discrepancy must therefore be regarded as evidence that the mechanism assumed for the exchange reaction is incorrect. The most likely alternative is that the reaction proceeds in two steps, with an enol of pyruvate formed as an intermediate. It seems probable that the enzyme-catalyzed decarboxylation and the non-enzymatic, Mn^{++}-catalyzed decarboxylation are alike in this respect (8, 18).

PROPERTIES OF THE ATP-DEPENDENT EXCHANGE REACTION: A series of experiments were carried out to determine the dependence of the exchange reaction on the components of the system. The affects of varying time of incubation, amount of protein, Mn^{++} concentration, and ATP concentration are shown in table III. When Mg^{++} was substituted for Mn^{++}, the effect varied with the enzyme source tested (table IV).

NUCLEOTIDE SPECIFICITY: After extensive purification of the pepcarboxykinase from avian liver, Utter and his collaborators were able to show that the reaction utilized ATP only indirectly (12, 22, 23). The natural substrate appeared to be GTP, though ITP was also active. Similarly, the pepcarboxykinase of lamb liver was shown by Bandurski and Lipmann (5) to use ITP in preference to ATP. The present studies included an examination of the nucleotide specificity of the ATP-dependent exchange catalyzed by enzyme preparations from several of the plant sources. Preparations from turnip were tested most extensively, with ATP, ITP and GTP. Representative preparations from five other sources were examined with ATP and ITP. The results are summarized in table V. In each experiment, the blank determination and the determination with added nucleotides were carried out with the same enzyme under strictly comparable conditions. The major conclusion was that in every one of the cases examined, ATP was much more effective than ITP. ATP was also considerably more effective than GTP with the turnip preparation. The previously reported finding (19) that ITP was as effective as ATP with wheat germ could not be confirmed. This may have been due to the fact that the enzyme preparations were very impure. It is possible that the discrepancy may be due to a transphosphorylation brought about by a trace of adenine nucleotide present in either the ITP or the enzyme preparation. In any event, it appears that ATP is most likely to be the natural substrate for the pepcarboxykinase from higher plants.

DISTRIBUTION OF PEPCARBOXYLASE: The spectrophotometric test described by Tchen and Vennesland (19) was used to test the various enzyme preparations for the presence of pepcarboxylase. This test is applicable only in the presence of malic dehydrogenase. It is based on the oxidation of DPNH by OAA formed from added PEP, the oxidation being measured by following the decrease in optical density at 340 mμ. The enzyme solutions were tested first to ensure that DPNH was rapidly oxidized by added OAA, but not by added pyruvate. All the enzyme preparations tested fulfilled this criterion. The effect of added PEP on the oxidation of DPNH was then determined. The enzyme preparations usually caused an oxidation of DPNH in the absence of added PEP. When this DPNH oxidation was rapid, the assay procedure was less reliable, but the use of careful controls often made it possible to conclude that the addition of PEP definitely increased the rate of oxidation of DPNH. In addition to spinach leaf and

TABLE V

NUCLEOTIDE SPECIFICITY OF EXCHANGE REACTION

ENZYME SOURCE	EXCHANGE ($10^{-2} \times$ CPM)			
	NO NUCLEOTIDE	ITP	ATP	GTP
Turnip	2.4	3.0	42	...
"	...	...	171	22
Corn seedling	2.3	6.3	90	...
Squash	3.1	9.0	83	...
Cauliflower bud	4.5	7.7	73	...
Lupine cotyledon	2.2	3.8	43	...
Wheat germ	2.9	2.2	10	...

The test system was the same as that of table I. Nucleotides were added as specified in the amount of 10 micromoles per 10 ml reaction mixture. The two experiments with turnip were performed with different enzyme preparations.

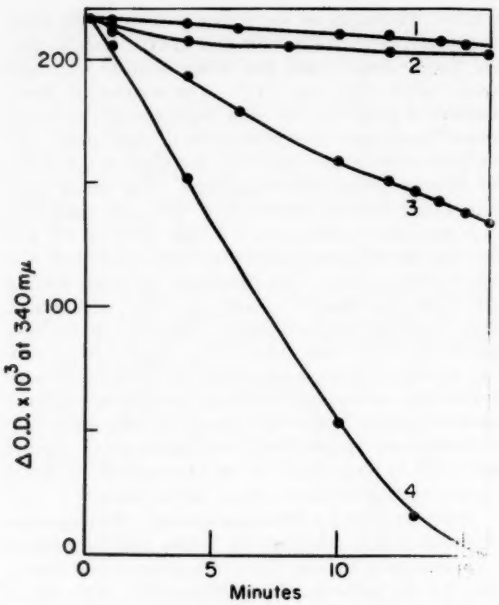

FIG. 1. The reaction mixtures contained 60 micromoles of phosphate buffer of pH 7.0, 10 micromoles of $MgCl_2$, 0.117 micromoles of DPNH, and other addenda as indicated, in a total volume of 3 ml. Each mixture contained 0.1 ml of the same extract from pea seed as was used in the experiment of table I. The reaction mixture was placed in cuvettes of 1 cm light path and the optical density at 340 mμ was measured with a Beckman spectrophotometer. The readings plotted on the graph have been corrected for absorption of all components other than the DPNH. Curve 1, no added substrate; curve 2, 2 micromoles of PGA and 30 micromoles of NaF; curve 3, 2 micromoles of PGA; curve 4, 2 micromoles of PEP. The curve for PEP and NaF could almost be superimposed on that for PEP without NaF.

wheat germ, which have previously been shown to contain pepcarboxylase, the following enzyme sources gave clearly positive tests: pea seed, parsnip root, cauliflower buds, cabbage leaf, turnips, lupine cotyledon, mustard leaf, and squash. With other sources tested, results were questionable or negative, but these negative results should not be regarded as definitive proof of the absence of the enzyme. In some of the cases where added PEP caused an oxidation of DPNH, added phosphoglyceric acid (PGA) was also tested, and found to be effective, but to a lesser extent than PEP. The oxidation of DPNH by PGA under these circumstances could be inhibited by NaF, but this reagent did not affect the oxidation of DPNH by PEP. These phenomena are similar to those already reported in detail for spinach and wheat germ. The inhibition by F^- is readily explained by the action of this inhibitor on enolase. A typical set of experiments with pea seed is shown in figure 1.

The DPNH oxidase activity of the extract from pea seed was smaller than that generally encountered in the various enzyme sources tested. Other data are not given in detail because of the general similarity of the results to those previously documented.

THE FORMATION OF C^{14}-OAA FROM PEP AND ADP: Pepcarboxylase will cause the formation of β-carboxy-labeled OAA (C^{14}-OAA) from PEP and $C^{14}O_2$, but pepcarboxykinase can cause C^{14}-OAA formation only if ADP is added with PEP. The dependence of the reaction on addition of both ADP and PEP can best be demonstrated in the absence of pepcarboxylase. Experiments which illustrate this are shown in table VI. The enzyme preparations used had been shown to be free of pepcarboxylase. The data in the table also show that pyruvate alone was inactive as a substrate, and that IDP could not be substituted for ADP.

Since no cycling is necessary when OAA is formed directly from PEP, CO_2 and ADP, the data of table VI should give a more realistic measure of the rate at which OAA may be formed by the pepcarboxykinase

TABLE VI

SYNTHESIS OF OAA FROM PEP AND $C^{14}O_2$

ENZYME SOURCE	ADDITIONS IN MICROMOLES	C^{14}-OAA (10^{-2} × CPM)
Turnip root	100 PEP, 50 ADP	236
" "	100 PEP	0.3
" "	50 ADP	0.3
" "	100 Pyruvate	0.2
Corn seedling	50 PEP, 100 ADP	140
" "	50 PEP, 100 IDP	3.4
" "	50 PEP	0.6

The reaction mixtures contained 500 micromoles of collidine buffer of pH 7.0, 100 micromoles of $MnCl_2$, $C^{14}O_2$ as in the "exchange" assay, 1 ml of enzyme, and other components as specified, in a final volume of 10 ml. Incubation was for 30 minutes at room temperature. After the reaction had been stopped by the addition of citric acid, 50 micromoles of OAA were added as carrier. The decarboxylation of OAA and the determination of the radioactivity of the CO_2 were then carried out exactly as in the "exchange" experiments previously described. The results, given in counts per minute, corrected for background, are comparable with those of the previous tables. The enzyme preparation from corn seedling contained 9.5 mg of protein per ml. The preparation from white turnips contained 7.3 mg of protein per ml. This preparation had been partially purified for the ability to catalyze the ATP-stimulated exchange reaction. The turnips were homogenized with phosphate buffer, the solids were removed by centrifugation and the activity was precipitated from the extract by addition of $(NH_4)_2SO_4$ to 60% saturation. The protein was dissolved in water, and the salt was removed by dialysis. A precipitate which formed when the solution was heated to 50° C for 5 minutes was removed by centrifugation. The pH was adjusted to 5.2 with acetic acid and a suspension of calcium phosphate gel was added (0.2 mg per mg protein. The gel was removed and the supernatant was fractionated with $(NH_4)_2SO_4$. The most active fraction was obtained between 35 and 50% saturation. It was dialyzed to remove salt, and lyophilized. The purification as measured by the ATP-stimulated exchange was 10-fold over the first extract, per mg protein.

reaction than any of the data obtained by measurement of the ATP-stimulated exchange. The latter test has the advantage, however, of being qualitatively more specific. Thus, pepcarboxylase will give no ATP-stimulated exchange reaction, but it will cause OAA formation from PEP and CO_2 alone, without added ADP. Under these circumstances, it is obviously difficult to determine a small amount of pepcarboxykinase. For this reason mainly, the ATP-stimulated exchange test was selected for use in the broad survey of the distribution of pepcarboxykinase. It is necessary to keep in mind, however, that even though pepcarboxylase alone can cause no ATP-stimulated exchange whatever, it can cause an increase in the magnitude of the exchange test observed if pepcarboxykinase is present. Any quantitative evaluation of the ATP-stimulated exchange test therefore requires consideration of the possible effect of the pepcarboxylase which may be present. It is possible, for example, that the relatively large stimulatory effect of Mg^{++} observed in the ATP-stimulated exchange catalyzed by a lupine cotyledon preparation (table IV) may be due to a relatively large amount of pepcarboxylase present, since pepcarboxylase appears to respond to Mg^{++} better than does pepcarboxykinase in those cases where the enzymes have been tested separately (3).

RESULTS WITH SPINACH: Spinach leaf had been shown in previous work by others (4) to give no ATP-stimulated exchange reaction, but to be an excellent source of pepcarboxylase. Because of the large amount of enzyme present and the absence of pepcarboxykinase activity, spinach appeared to be well suited for a study of the possible localization of pepcarboxylase. Previous work with spinach had all been done with extracts of acetone powders. Since acetone treatment disrupts particulate structures, it could not be employed in the localization studies. Solubles and particulates were therefore separated by centrifugation, as described in the section on Materials, and protein-containing extracts from the various fractions were tested for catalysis of labeled OAA formation from PEP and $C^{14}O_2$ in the presence and absence of ADP. Note that in all cases a protein solution was used for the experiment. No chlorophyll was present in the incubation mixtures.

The results given in table VII show that pepcarboxylase is associated with the particulate fraction and that it is not removed by repeated washing of the particulates in the suspending medium used, though the enzyme is readily solubilized after lyophilization. The small particulate fraction which consisted of mitochondria and chloroplast fragments, was more active per mg protein than the intact chloroplast fraction, but the intact chloroplasts contained considerable activity. The question of whether the enzyme is really present in the chloroplast or in smaller particulates, or in both, should probably be left open, however, since it is difficult to determine the extent to which the chloroplasts are contaminated by mitochondria (which have been shown to be present in leaves (15)), and since the smaller particulate fraction certainly contained a high proportion of chloroplast fragments. The washing procedure removed protein from both fractions, but most of the enzyme activity was retained, with the result that the activity per mg protein was considerably increased by washing.

TABLE VII

INTRACELLULAR LOCALIZATION OF ENZYMES IN SPINACH LEAF

PREPARATION TESTED	MG PROTEIN	C^{14}-OAA (10^{-2} × CPM)		SPECIFIC ACTIVITY *	
		50 MICROMOLES PEP	50 MICROMOLES PEP, 100 MICROMOLES ADP	50 MICROMOLES PEP	50 MICROMOLES PEP, 100 MICROMOLES ADP
Total particulates	4.7	38	100	8.1	21
Cytoplasm	17.7	0.05	0.09	0.003	0.005
Intact chloroplasts	5.8	5	20	0.9	3.4
Small particulates	5.6	54	147	9.7	26
Washed chloroplasts	1.7	29	59	17	35
Washed small particulates	1.9	98	230	52	121

The procedure was the same as that described for table IV.

* Specific activity = $\frac{10^{-2} \times \text{cpm}}{\text{mg protein}}$

The surprising fact which emerged from these experiments was that ADP caused a considerable stimulation of the β-carboxyl-labelled OAA formation. This was not expected because of previous failure to detect pepcarboxykinase in spinach. The simplest interpretation of the unexpected ADP effect was that pepcarboxykinase was indeed present, and that previous failures to detect it were due to the inactivation of the enzyme by the different preparative procedures previously employed. The spinach particulates were therefore tested for their ability to cause an ATP-stimulated exchange reaction. The results were negative. Because of the anomalous nature of these results, the experiments were repeated many times. The ADP effect was readily duplicable. Two- to four-fold stimulation of C^{14}-OAA formation was obtained with fresh particulate preparations, but no ATP-stimulated exchange reaction was observed. Table VIII shows the results of experiments performed to test the variation of the effect with the amount of ADP added, and the effect of IDP. It is apparent that IDP is as effective as ADP, in contrast to the results obtained with the other plant sources. Elucidation of the nature of the ADP effect observed with spinach requires further experimentation, with a tentative conclusion that the effect cannot be attributed to a pepcarboxykinase of the sort demonstrated and studied in other plant sources.

Localization Studies with Cauliflower and Pea: A few preliminary experiments were carried out with cauliflower bud homogenate and with pea seedlings, to see whether the enzymes could be localized in the soluble or particulate fractions. Both of these sources contained both pepcarboxylase and pepcarboxykinase activity. The question of localization is of interest, because a mixture of the enzymes would behave like an ATP-ase. That is, the net effect of the two enzymes acting together (with a small amount of either PEP or OAA) would be to convert any ATP which was present to ADP and inorganic phosphate. It would be interesting to know whether the cell actually employs such a mechanism for hydrolyzing ATP.

Efforts to show a completely separate localization of the two enzymes in solubles and particulates of cauliflower buds and pea seedlings were not successful. The results obtained with the fractions from cauliflower are given in table IX. Each fraction was tested for its ability to cause labelled OAA formation from PEP alone, from PEP and ADP, and from OAA in the presence of ATP. The latter tests were carried out as described for the assay procedure of table I. The other experiments were carried out as described for the experiments of table VIII. The data of table IX shows that no clear-cut separation of the enzymes was achieved by the centrifugation procedures employed, and that the cytoplasmic protein appears to be richer in both enzymes. In the case of pea seedling, there was a higher ATP-stimulated exchange in the particulate protein fraction than in the soluble protein. Thus, the standard "exchange" assay gave a value of 8.4×10^2 cpm for 6.5 mg of mitochondrial protein, and 4.6×10^2 cpm for the same amount of cytoplasmic protein. The latter result is close to the blank level, but is not clearly negative.

Discussion

One of the main questions pertinent to function is the problem of the rate at which the carboxylases operate to form OAA. A measure of this rate is provided by the experiments in which the enzyme preparation is incubated with PEP and $C^{14}O_2$, with or without ADP. The data given as counts per minute of C^{14}-OAA can be converted to micromoles of OAA formed per unit time for a given amount of enzyme preparation by multiplying the cpm for C^{14}-OAA by $50/3 \times 10^6$. (50 micromoles of bicarbonate with a thick sample count of 3×10^6 were present in the incubation mixture, and the CO_2 was collected with 50 micromoles of carrier OAA. One may assume that the amount of OAA formed is inappreciable in relation to the amount of carrier added. Though this is not strictly correct, the error introduced by the assumption is probably no greater than the errors of the measurements.) Thus, for corn seedling (table VI), $14{,}000 \times 50/3 \times 10^6 = 0.23$ micromoles of C^{14}-OAA was formed in the incubation period of 30 minutes. The present studies were not planned to obtain accurate rate measurements, and the figures should only be regarded as approximate. There is also no assurance that test conditions were optimal, or that the preparation of the extracts did not entail a loss of activity.

Carboxylase and carboxykinase may both be regarded as enzymes catalyzing steps in the conversion of carbohydrate to malate. The well-known crassulacean acid metabolism of the succulents has been shown to consist of the production of malic acid at night with a diminution of carbohydrate followed by a loss of malate in daylight with an increase of carbohydrate (20). It has been suggested that this diurnal fluctuation is not confined to the succulents, but is widespread throughout higher plants, the difference between succulents and non-succulents being quantitative rather than qualitative (21). Saltman et al (16) have identified OAA as the probable first product of dark CO_2-fixation in leaves of *Bryophyllum calycinum*, and have demonstrated the presence of pepcarboxylase in leaf extracts. The present work shows that both pepcarboxylase and pepcarboxykinase are widely distributed.

Table VIII

ADP Effect with Spinach Particulates

Expt. no.	Nucleotide	C^{14}-OAA ($10^{-2} \times$ cpm)
1	.....	500
	10 micromoles ADP	630
	50 micromoles ADP	980
	100 micromoles ADP	1050
2	.....	210
	100 micromoles ADP	570
	100 micromoles IDP	490

The reaction mixtures and procedures were the same as those described for table VI, except that 50 micromoles of PEP were added in every case and the nucleotides were added as indicated. In experiment 1, each reaction mixture contained 3.4 mg of protein from small particulates. In experiment 2, each reaction mixture contained 3.8 mg of protein from another preparation of small particulates. The small particulates were prepared as described for the experiments of table VII.

Table IX

Results with Cauliflower Bud Homogenate

Enzyme source	Additions in micromoles	C^{14}-OAA ($10^{-2} \times$ cpm)
Mitochondria	100 PEP	20
	100 PEP, 50 ADP	64
	100 OAA, 10 ATP	6
Cytoplasm	100 PEP	186
	100 PEP, 50 ADP	254
	100 OAA, 10 ATP	19

The experiments with mitochondria were carried out with 3.8 mg of protein, and those with cytoplasm were carried out with 7.3 mg of protein. The reaction mixtures and procedures for the experiments with PEP alone and with PEP and ADP were the same as described in the legend for table VI. The experiments with OAA and ATP were carried out as described for table I.

The OAA formed by carboxylation would not be expected to accumulate. One of the most active enzymes in plant tissues is malic dehydrogenase, and any OAA formed would be immediately reduced to malate if reduced diphosphopyridine nucleotide (DPNH) were available. An unpublished study has been made in our laboratory by A. Magaldi, of the relative rates of oxidation of DPNH by various oxidants in the presence of acetone powder extracts from a variety of plant sources. The results with OAA, calculated as micromoles DPNH oxidized per hour per mg dry weight of the extract, may be listed as follows: pea shoot, 90; pea root 210; pea seeds 28, 36; parsley leaf 31, 30, 11.5; cress shoot, 101; carrot root 11; wheat seedling root, 31; and wheat germ, 220. Oxidation of DPNH by pyruvate never approached such activities, and the oxidation of DPNH by hydroxypyruvate in the presence of leaf extracts, which is a rapid reaction (17), was never more than 15 % as rapid as the oxidation by OAA.

In connection with the spectrophotometric assays of pepcarboxylase described in the present paper, measurements were always made of the rate of oxidation of DPNH by OAA and by pyruvate in the presence of the various extracts tested. These tests showed that OAA always oxidized DPNH at a rate much more than sufficient to account for the reaction observed with added PEP, whereas the oxidation of DPNH by pyruvate was always too slow to account for the PEP effect observed. The results all confirmed the generalizations made from the earlier observations of Magaldi, that malic dehydrogenase activity is always high, and lactic dehydrogenase activity is low or absent. In many instances, where DPNH is oxidized by addition of pyruvate, it is possible that the pyruvate is first decarboxylated to acetaldehyde which then acts as oxidant for DPNH. (The relatively high lactic dehydrogenase content of potato appears to offer an exception to the general rule (17).

The data available on enzyme activities therefore suggest that in many, if not all, plant tissues, malate should be regarded as the natural end-product of the anaerobic phase of carbohydrate breakdown, analogous to lactate in muscle and ethanol in yeast.[5] (This does not imply that lactate and ethanol formation have no functional significance in higher plants, but only that malate formation is more important quantitatively in most cases. Lactate formation appears to be of minor significance, with ethanol formation occupying an intermediate position.) Since many plants contain a malic enzyme (24) in addition to pepcarboxylase and pepcarboxykinase, the interplay of all three CO_2-fixing reactions must be taken into account in any description of the course of molecular events within the intact tissue. The three CO_2-fixing enzymes must have different functional roles. It has been pointed out previously (19) that pepcarboxykinase can cause a conversion of OAA to PEP and so may act in the synthesis of carbohydrate from organic acids, whereas pepcarboxylase cannot function in this way. Furthermore, since the direction of the pepcarboxykinase reaction can be determined by the concentration levels of CO_2, ADP, and ATP, it is not difficult to visualize a means whereby the photosynthetic production of ATP and utilization of CO_2 could lead to malate disappearance, whereas increase in CO_2 and conversion of ATP and ADP would lead to malate formation. The pepcarboxylase reaction, on the other hand, could account for the conversion of carbohydrate to malate at low CO_2 tensions, and would not respond to changes in the concentration levels of ATP and ADP. Nevertheless, the fact that these various enzymes have different functional potentialities does not completely define their relations to each other, and the manner in which their action is integrated within the cell is a subject worthy of further investigation.

[5] If malate formation does not occur anaerobically, but requires O_2, then the formation of PEP cannot occur over the classical path from fructose-1,6-diphosphate by way of the aldolase and triose-phosphate dehydrogenase reactions, which would provide adequate amounts of DPNH.

Summary

Assay procedures have been described for detecting pepcarboxylase and pepcarboxykinase in the tissues of higher plants, with special attention to the problems encountered in assay when the two enzymes are present in the same extract. Both enzymes were shown to be widely distributed in plant tissues. They frequently occur in the same source. The pepcarboxykinase of higher plants is specific for ATP rather than other nucleotides. Anomalous results with spinach particulates showed that OAA formation from PEP was enhanced by ADP and IDP, though other tests for pepcarboxykinase were negative. The pepcarboxylase of spinach was localized in particulates. In cauliflower buds and pea seedlings, both the mitochondrial and the cytoplasmic proteins appeared to contain both enzymes, though in different amounts.

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GROWTH REGULATORS AND FLOWERING. I. SURVEY METHODS[1,2]

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Recent investigations have shown that applied auxin may have either a positive or a negative effect upon the flowering of certain plants (e.g., 3, 7, 9, 14, 16, 22, 23, 26, 29, 32, 35, 36). Growth regulators other than auxin (e.g., maleic hydrazide (18, 30), triiodobenzoic acid (12), gibberellin (21, 37), cobaltous ion (24), etc.) have also been shown to influence the flowering process in a number of plants. On the basis of the auxin effects, theories of the mechanism of floral induction have been proposed (1, 6, 10, 19, 20, 24, 25, 31), and additional insight into the partial processes of induction has been gained (27, 33).

Specifically, the present work was carried out to answer the following four questions: 1) Does a particular growth regulator influence flowering? 2) Which phase of the flowering process does it influence? 3) Will effects upon flowering illuminate the flowering mechanism? 4) Will effects upon flowering illuminate the mechanism of action of a given growth regulator upon growth?

Seven compounds were chosen as representatives of growth regulator types presently considered to be of importance: maleic hydrazide (MH), 2,2-dichloro-

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propionic acid (dalapon), 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA), 3-indoleacetic acid (IAA), cobaltous ion (Co^{++}), and 2,4-dinitrophenol (DNP). A few other compounds were investigated in a preliminary way while others await study by the methods described below.

Methods

Plants of *Xanthium pennsylvanicum* Wall. were grown, as previously described (3, 32), from seed in pint-sized plastic containers in a rich soil with a small amount of commercial fertilizer (Vigoro) added at an early stage. Seeds were supplied by James Bonner at the California Institute of Technology. The plants were maintained in the vegetative condition until the time of treatment by extending daylength with incandescent light (ca 50 ft-c) to make a total day length of 17 to 21 hours. Plants were defoliated to a single leaf (usually the most actively growing one) just before treatment. Except in the critical night length experiments, plants were induced to flower with a single dark period by placing them in six light-tight cabinets for 16 hours (little temperature control). Growth regulators were applied by dipping the upper four or five inches of the shoot, including the one remaining leaf, into 400 to 1,000 ml of solution containing growth regulator and about six drops of Dynawet (Dow Chemical Company) per liter. The plants were selected before each experiment so that each treatment (containing 7 to 20 replications) covered the range of environmental conditions in the greenhouse, and after induction plants were systematically set out on the bench so that each treatment formed a series of diagonal lines extending through the entire experiment. Thus, essentially the same range of variation was introduced into each treatment and, because of the systematic design, detailed statistical analysis was not attempted. Differences discussed in this paper are, however, of such magnitude that they may be regarded with confidence. When growth regulators were applied during the dark period, a very weak green light was used and, since treatments were in separate cabinets, individual replications were subjected to the green light only once during the dark period.

A number of days (usually 9) after induction apical buds were examined under a dissecting microscope (36× magnification) and classified according to a series of previously described floral stages (32). Floral stage is roughly proportional to time after induction (the development control points of figs 9 to 15) and to length of the dark period (control points of fig 16).

Three general types of experiments were carried out using these methods. It will be convenient to describe these experiments in the section which presents their results.

Data concerning date of experiment, number of replications, leaf left after defoliation, and number of days from induction to examination of apical buds, are given for each experiment in table I.

Table I

Data Relating to Experimental Conditions for Results Shown in Figures 1 to 16 and Table II

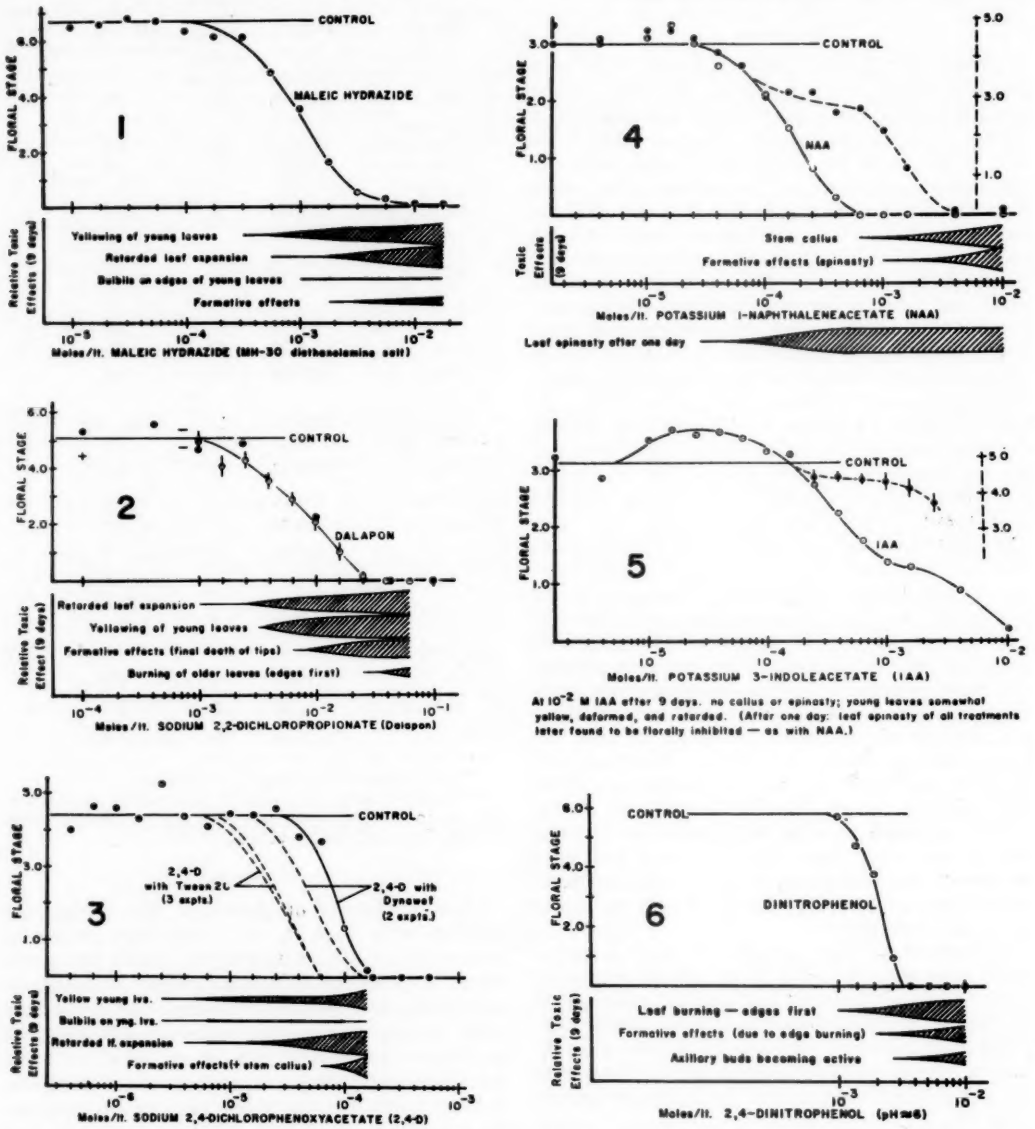
Fig. no.	Date *	Plants per treatment	Leaf left on plants **	Days to dissection
1. MH	4/27/56	10	#3	9
2. Dalapon				
Open circles	8/3/56	16	#3	8
Solid dots	7/16/56	10	#3	9
3. 2,4-D				
Open circles	6/5/56	11	#3	9
4. NAA				
Open circles	6/13/56	10	#3	9
Solid dots	9/11/56	10	T-#3	9
5. IAA				
Open circles	7/5/56	15	#3	9
Solid dots	9/14/56	20	#3	9
6. DNP	8/7/56	15	#3	9
7. Co^{++}				
Open circles	6/13/56	10	Large #3	9
Solid dots	9/3/56	10	#3	9
9. MH	7/30/56	7	T-#3	7
10. Dalapon	8/2/56	8	T-#3	9
11. 2,4-D	7/28/56	7	T-#3	10
12. 2,4-D, IAA	8/8/56	10	7 Plants T-#3 3 Plants Small #3	9
13. NAA	8/6/56	8	T-#3	9
14. DNP	11/5/56	10	#3	9
15. Co^{++}	8/15/56	10	T-#3	9
16. Co^{++} + 2,4-D	8/11/56	10	T-#3	9
Table II	11/16/56	10	Small #3	9

* Date plants were placed in cabinets.
** Numbers refer to length of leaf midrib as follows: #3, 5.9 to 9.2 cm; T-#3, 6.9 to 8.5 cm; small #3, 5.9 to 7.7 cm; large #3, 7.7 to 9.2 cm.

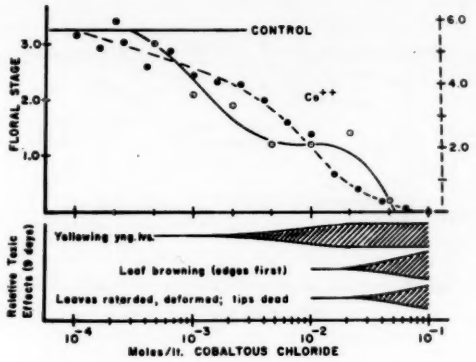
Results

In the first type of experiment, growth regulator solutions were made up in a logarithmic concentration series and applied to prepared plants just previous to the single inductive dark period. If the plants were visibly affected at any concentration, notes were taken the day after growth regulator application, and in all cases notes were taken at the time buds were examined (usually 9 days after induction). In figures 1 to 7, results with the seven compounds mentioned above are shown, and figure 8 shows the solid lines of the other figures compared on a single concentration scale. Flowering condition is shown as floral stage plotted against concentration of growth regulator on a logarithmic scale, and effects of growth regulators upon vegetative growth are shown by shaded bars with a subjective description of the visible effects as compared with controls. Width of the bar is pro-

portional to extent of toxic response at a given concentration. Since the effects varied considerably with different compounds, no attempt was made to develop a more objective system of measuring these effects and notes taken were purely descriptive and subjective. It should be noted from the figures that the auxins may inhibit flowering without causing any noticeable formative effects, while the other compounds produce such effects at the same concentrations at which flowering is inhibited.



FIGS. 1 to 6. Flowering response of plants to a concentration series of growth regulators (the curve in the upper part of each figure) as compared with visible effects of growth regulators upon the plants (the cross-hatched bars in the lower part of each figure). Vertical lines through the points in figures 2 and 5 represent standard error. Open circles refer to the scale of floral stages on the left ordinate, and in figures 4 and 5, solid dots and broken lines refer to floral stages on right ordinate. Right ordinates were adjusted so that controls of both experiments would coincide. Broken lines in figures 3, 4, and 5 indicate that results may vary greatly between experiments, and hence the curves as shown have no absolute significance. Formative effects refer to twisted or misshapen leaves, petioles or stems. Data for experiments given in table I.



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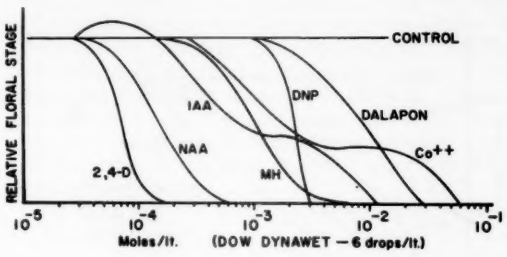

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FIG. 7. Flowering response of plants to a concentration series of cobaltous ion as compared with visible responses of the plant after nine days. Solid dots refer to right ordinate.

FIG. 8. The solid curves of figures 1 to 7 placed on the same concentration scale for comparison.

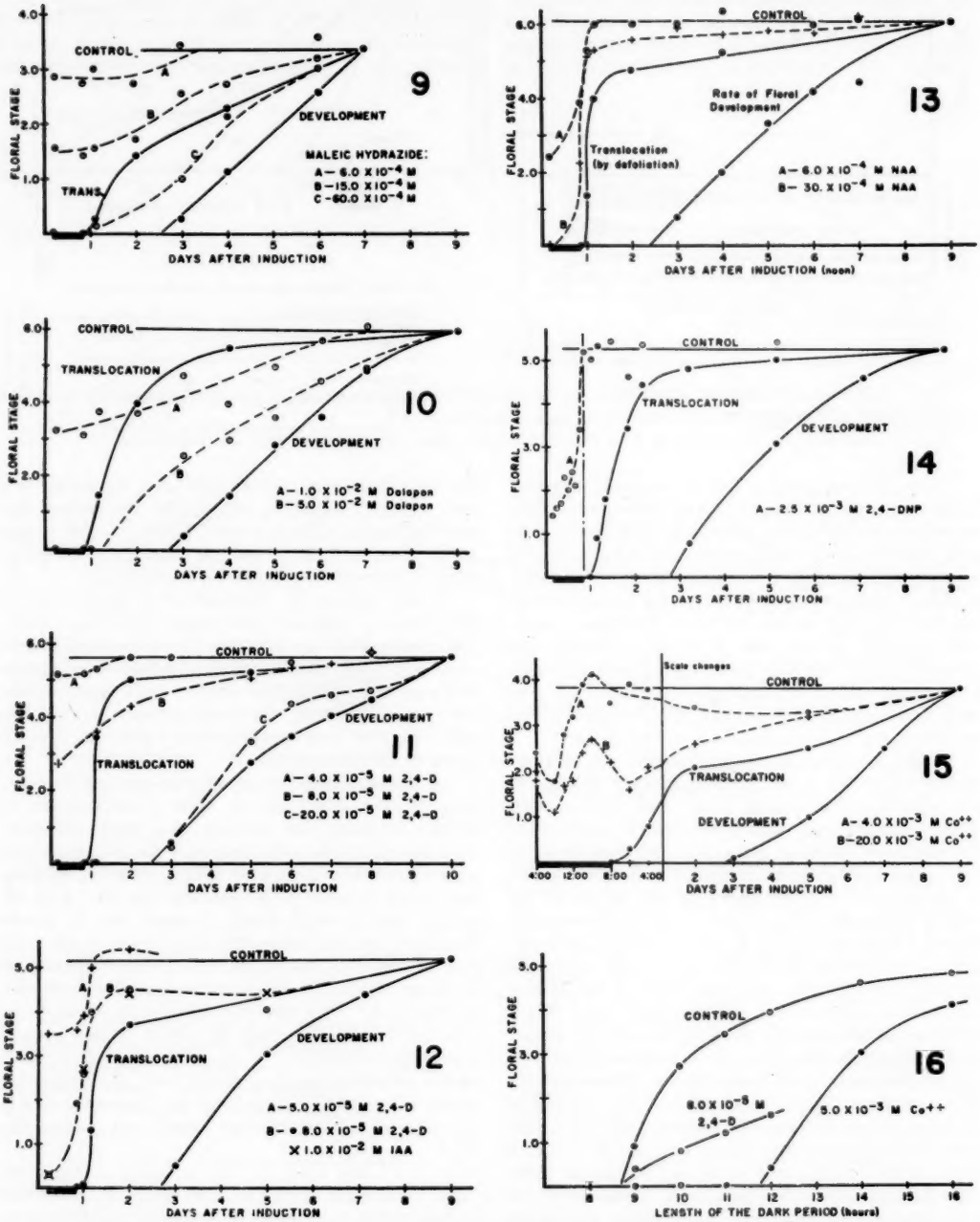
Some other compounds were investigated in a very preliminary way by the first type of experiment. Concentrations of 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} molar were used. Sodium diethyl dithiocarbamate and beta-methyl umbelliferone seemed to have no effect on flowering at these concentrations. At herbicidal concentrations, the following compounds inhibited flowering: pentachlorophenoxy acetic acid (10^{-2} M), N-meta-tolyl phthalamic acid (10^{-2} M), and 2,4,6-tribromophenoxyacetic acid (10^{-3} M). Coumarin appeared to promote flowering at 10^{-2} M, but the experiment was inconclusive.

In the second type of experiment, one or more concentrations of growth regulator were applied to groups of plants at various times from the beginning of the inductive dark period, through the dark period and the days following induction until two days before apical buds were examined. The data are shown in figures 9 to 15. In assessing the effects of the growth regulators, three types of controls were considered. First, one group of plants received no treatment at all. This control is shown as a horizontal line and a solid dot at the upper right in the figures. Its position indicates the number of days after induction that all buds except those of the second control were examined. Second, plants were examined at various intervals after induction to determine the course of floral bud development. This control is shown as a solid line usually beginning 2.5 days after induction and extending upwards to the first control point. Third, plants were defoliated at various times after induction and then buds were examined at the time indicated by the first control point. Assuming that floral stage at a given time after induction is a measure of amount of flowering hormone reacting at the tip (27, 32, 33), the defoliation control gives a measurement of the amount of flowering hormone translocated out of the leaf at the time of defoliation (17, 33). This control is shown by a solid, S-shaped line extending from a short time after induction to a near maximum reached usually by the second day after induction. Floral stage of plants treated with a given concentration of growth regulator at various times is shown in the figures as a broken line.

It can be seen by examination of figures 9 to 15 that dalapon, MH and 2,4-D inhibit flowering regardless of when they are applied; the auxins inhibit flowering only when they are applied before translocation of flowering hormone is complete; DNP inhibits only when applied during the dark period; and Co^{++} is most effective when applied during the first 8 or 10 hours of the dark period.

In the third type of experiment, groups of plants were treated with one or more concentrations of growth regulator just previous to a single inductive dark period which varied in length for different treatments from 9 to 16 hours. Floral stages of controls and plants treated with cobaltous ion or 2,4-D are plotted against night length in figure 16. It is evident that 2,4-D depresses the floral stage at all night lengths but does not change the minimum period of darkness required to bring about flowering (in fig 16, ca. 8⅔ hrs). This had previously been shown to be true for NAA (33). All the above named growth regulators were tested in this way, and all of them except cobaltous ion gave curves very similar to that produed by 2,4-D. Cobaltous ion, however, clearly increased the length of the critical dark period (fig 16).

It was noted from the data in figure 14 that in all DNP treatments which inhibited flowering, DNP was applied either just before or while the plants were in darkness. Thus it appeared possible that penetration of herbicide or some other factor might be a function of light, and that the effects upon flowering per se might be more apparent than real. To test this, DNP was applied to plants before and after an inductive dark period as before, and then two days later in-



FIGS. 9 to 15. Flowering response of plants treated with growth regulators at various times in relation to a single inductive dark period. Solid dots and lines represent controls as explained in the text.

FIG. 16. Effect of 2,4-D and cobaltous ion upon critical night length. Data for all experiments given in table I.

TABLE II

EFFECT OF LIGHT ON DNP TOXICITY

TREATMENT	FLORAL STAGE	DAMAGE TO PLANTS
1. Plants induced Nov. 16, 1956 with a 16-hr night, no further treatment.	3.3	None
2. Plants treated with 2.5×10^{-3} M, DNP just before induction.	1.1	Leaf edges burned, formative effects.
3. Plants treated with 2.5×10^{-3} M, DNP just after induction.	3.5	None
4. Treated with DPN Nov. 18, 1956, 1:05 P.M. left in light.	3.1	None
5. Treated with DNP Nov. 18, 1956, 1:05 P.M. 4 hrs dark.	3.8	Leaf edges burned, formative effects.
6. No treatment except induction; 4 hrs dark on Nov. 18, 1956.	3.8	None

duced plants were treated with DNP and given either a 4-hour dark period or left in the light. Control plants were given the 4-hour dark period without DNP treatment. The results are shown in table II. It is evident that the short period of darkness following DNP treatment two days after induction had no effect upon flowering. Yet it is also evident from the data indicating plant damage that toxic effects of DNP are wholly dependent upon a period of darkness following application of the chemical.

DISCUSSION

It is convenient to consider the above results in light of the questions put forth in the introduction.

1. Does a particular growth regulator influence flowering? As shown by the concentration curves (figs 1 to 8), all of the growth regulators used in these experiments do influence flowering, and all of them at one concentration or another reduce the degree of flowering observable nine days after induction. It would appear that the first type of experiment provides a simple and effective method of screening growth regulators for their effects upon flowering. There are possibly three drawbacks. First, it is conceivable that a compound might influence flowering when applied at some time other than just before induction, but not influence flowering when applied at this time. No such instances are known, although auxin and Co^{++} may be slightly more effective in the inhibition of flowering when applied some 2 to 3 hours after the beginning of induction than when applied just before the plants are placed in the dark (32, 33, fig 15). Second, since plants are induced with a dark period which brings about almost maximum induction, promotion of induction or floral development might be difficult to detect. Such a promotion has been reported for low concentrations of auxin (38), and is perhaps evident for IAA in the solid line of figure 5 (although statistical significance is questionable), and for NAA in figure 4. Such a promotion might be easier to observe by using a short inductive dark period or by using some other criterion of flowering (39) in addition to floral stage. Third, de Zeeuw has recently shown that flowering response to applied chemicals depends upon age of the leaves on the plant (38). In the above experiments, only the most rapidly expanding leaf was left on the plants at time of treatment.

2. Which of the partial processes of flowering are influenced by a particular growth regulator and what is its mechanism of action? The reactions of the photoinductive dark period, as postulated by Salisbury and Bonner (33), may be incorporated into the scheme of partial processes of photoperiodic induction for short-day plants as suggested by Bonner and Liverman (1) and by Liverman (24):

I. The High-Intensity Light Process (probably photosynthesis).
II. The Reactions of the Photoinductive Dark Period.
  1. Pigment Conversion. (The spontaneous conversion, within the first 2 to 3 hours of the dark period, of a photo-receptor pigment from a far-red-receptive to a red-receptive form.)
  2. Preparatory Reaction(s). (Reaction following Pigment Conversion which is an essential part of the mechanism which determines the critical night length.)
  3. Hormone Synthesis. (Synthesis in the leaf of the flowering hormone.)
III. Translocation of Flowering Hormone from the Leaf.
IV. Differentiation of Floral Primordia.
V. Development of the Flower.

Under certain conditions a complication arises in that a destruction of flowering hormone may take place towards the end of prolonged dark periods. Experiments designed to investigate this have led Lockhart and Hamner (27) to postulate a stabilization of flowering stimulus by high intensity light following the inductive dark period and preceding translocation. Liverman (24) has called this the Second High-Intensity Light Process.

Of the three kinds of experiments discussed in this paper, only the time of application and the critical night experiments can give clear answers relating to which partial process might be influenced by a given growth regulator. The concentration series type of experiment may even be misleading. Of the seven compounds tried, only the auxins inhibit flowering at concentrations which do not cause toxic effects, and even at these concentrations they cause a general leaf epinasty which disappears within a few days (figs 1 to 7). Thus one might suspect that the other five compounds inhibit flowering only by inhibiting growth in general. Yet the time of application experiments demonstrate that this is true only for MH and dala-

pon, which inhibit flowering regardless of when they are applied.

The time of application experiments are quite unambiguous if a compound is acting upon development of the floral bud, and they also show clearly if a compound is effective against flowering during the period before translocation of flowering hormone is complete. In combination with the critical night experiments, these tests will show if a compound is acting on the mechanisms controlling critical night or on synthesis of flowering hormone. A specific effect upon differentiation of floral primordia might also appear in such experiments. The experiments discussed in this paper do not, however, distinguish between an effect upon Pigment Conversion and an effect upon Preparatory Reactions, nor do they tell whether a compound is acting upon translocation or the Second High-Intensity Light Process. The experiments could probably be set up to study the First High-Intensity Light Process. It should also be noted that an effect upon development, translocation, etc., might mask effects upon all earlier processes except the critical night, which can be studied separately.

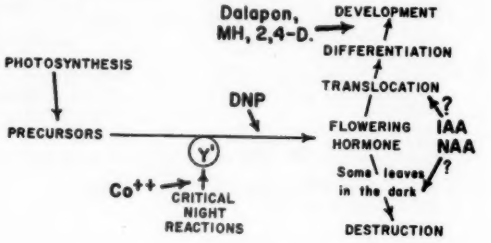
Of the seven compounds studied, MH and dalapon inhibit development of the floral bud, and 2,4-D is also an effective inhibitor of floral development. As has been previously shown, the auxins inhibit flowering only when they are applied before translocation of the flowering hormone from the leaf is complete (32, 33). This is again shown for NAA in figure 13. It would appear then, that auxin might inhibit translocation of flowering hormone or cause a destruction of flowering hormone in the leaf in either light or darkness (33). Experiments presently in progress suggest that the translocation hypothesis is least likely.

It can be seen from figure 13, that even NAA, when applied at a relatively high concentration causes some inhibition of floral bud development regardless of when it is applied. The concentration in this case (3.0×10^{-3} M) was high enough to also cause herbicidal effects (fig 4). All compounds used in these experiments inhibit development at sufficiently high concentrations.

DNP inhibits flowering only when applied before or during the period of hormone synthesis (fig 14). Since this compound is known to reduce the level of high energy phosphate available for reactions of synthesis, etc. (2, 15), this result might be expected. Yet it is not clear how translocation of flowering hormone, growth of the bud, etc., can proceed so well when high-energy phosphate has been lowered. This problem would seem to deserve intensive future investigation.

Cobaltous ion is effective only when applied during and slightly after the critical dark period (fig 15), suggesting that it influences this phase of flowering. The critical night length experiments (fig 16) bear this out. It is also interesting to note the fluctuations of effectiveness of 2.0×10^{-2} M Co^{++} applied at various times. This is reminiscent of the endogenous rhythms of Bünning (5).

The above partial processes and the effects of the seven growth regulators used in these experiments upon these processes can be summarized in the following diagram. The two phases of the critical night reactions are combined.



3. Will the effects of growth regulators upon flowering tell us more about the partial processes of the flowering mechanism? In the case of MH, dalapon and 2,4-D, the strong inhibitory effect upon bud development masks any other potentially interesting effects. The auxins have already led to further clarification of the partial processes (27, 33), and the results with Co^{++} and DNP are very promising.

The fact that the results presented here fit into the system of partial processes outlined above seems to be evidence in favor of the validity of that system. Results with Co^{++} and DNP seem to clearly separate the critical night reactions from Hormone Synthesis. The first two reactions of the dark period (Pigment Conversion and Preparatory Reaction), however, are not clarified by these experiments, although future work with Co^{++} might further illuminate this phase of the flowering process in cocklebur.

4. Will the effects upon flowering tell us more about the general mechanism of action of a particular growth regulator upon growth as well as flowering? In the case of maleic hydrazide, its postulated action as a general growth inhibitor seems to be confirmed (18), and dalapon seems to fit into this same category (not to imply that dalapon and MH both inhibit growth by the same mechanisms).

The results with 2,4-D are especially interesting in light of this fourth question. It is generally assumed that the herbicidal action of 2,4-D is related to its activity as an auxin (8). The results reported in this paper tend to raise doubts about this concept, although they do not refute it. The pattern of auxin effects upon flowering is quite clear, yet this pattern is not produced by 2,4-D except at very low concentrations, and most of the effects of 2,4-D upon flowering are of the type produced by MH and dalapon. If the herbicidal activity of 2,4-D were related to its auxin activity, and toxic effects appeared only at relatively high concentrations (in the range of bi-molecular attachment as postulated by Foster et al, 11), then one might expect that flowering would be inhibited only at concentrations much lower than those producing herbicidal effects, as is the case with the other auxins. However, the first perceptible sign of

floral inhibition is at a concentration which inhibits growth in general and causes severe toxic responses.

The striking effects with cobaltous ion must await further investigation before its mechanism of action can be elucidated. Possible clues to the problem appear in the literature: Miller (28) reported that cobaltous ion will replace the light requirement for expanding leaf discs (as will kinetin 34); and Galston and Siegel (13) report that cobaltous ion inhibits the IAA oxidase system. (This might suggest that applied cobaltous ion should result in a higher native auxin level, which would result in an inhibition of flowering. Yet it is difficult at present to relate the results with cobaltous ion in the above experiments to those obtained by using auxin.)

Little is known about the mechanism of action of dinitrophenol as an herbicide, although it is known to interfere with the oxidative phosphorylation mechanism (2, 15).

The data of table II indicate that DNP will cause damage to plants only if application of the chemical is followed by a period of darkness and that flowering is not inhibited by DNP which is applied after hormone synthesis is complete, even though damage to plants may be severe (the same is true for Co^{++}). This preliminary observation would seem to hold considerable promise of illuminating to some extent the mechanism of toxicity of dinitrophenol herbicides. For instance, the concentrations used in the experiment may inhibit oxidative phosphorylation but not photosynthetic phosphorylation. The level of ATP within the tissue would then be lowered by DNP only in the absence of photosynthesis. Perhaps such a lowered ATP level would account for the observed plant damage. This hypothesis is being tested at present.

Summary

1. Cocklebur plants were treated with a series of growth regulators just before being induced to flower by a single 16-hour dark period. After nine days notes were taken on the macroscopic condition of the plants, and apical buds were classified according to a series of stages of floral bud development. It was found that dalapon, maleic hydrazide (MH), 2,4-D, 2,4-dinitrophenol (DNP), and cobaltous ion inhibited flowering at concentrations which also caused a general inhibition of vegetative growth or other toxic responses, while indoleacetic acid (IAA) and naphthaleneacetic acid (NAA) inhibited flowering at concentrations which caused no visible effects on the plant.

2. Growth regulators were applied to plants at different times in relation to a single 16-hour inductive dark period. It was found that dalapon, MH, and 2,4-D would inhibit flowering regardless of when applied (i.e. they would inhibit development of the floral bud). IAA and NAA inhibited flowering only when applied before maximum concentration of flowering hormone had been built up outside the leaf (as shown by defoliation experiments). DNP inhibited flowering only when applied during the inductive dark period, and cobaltous ion inhibited flowering only when applied during the first eight to ten hours of the inductive dark period.

3. Growth regulators were applied just before plants were induced to flower with varying lengths of a single dark period. All of the above seven compounds inhibited flowering at all night lengths, but only cobaltous ion changed (increased by as much as three hours) the minimum period of darkness required to cause the first perceptible signs of flowering (the critical night).

4. On the basis of these data it is suggested that the growth regulators used in these experiments inhibit flowering by blocking or otherwise influencing the steps of photoperiodic induction in cocklebur as follows: MH, dalapon, and 2,4-D inhibit development of the floral bud. IAA, NAA, and low concentrations of 2,4-D most likely cause a destruction of flowering hormone in the leaf in either light or darkness. DNP inhibits synthesis of flowering hormone. Cobaltous ion interferes with the mechanism which controls the critical night length.

5. Results with 2,4-D indicate that its auxin effects might be separable from its herbicidal effects. Results with DNP show that the herbicidal effects of this compound on cocklebur depend upon a period of darkness following application of the chemical, although development of floral buds is independent of this response.

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AN ANALYSIS OF TRANSLOCATION IN THE PHLOEM OF THE BEAN PLANT USING THO, P^{32}, AND C^{14} [1,2]

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A description of translocation in quantitative terms has been delayed for lack of suitable techniques for its measurement. The passage of minute amounts of material through narrow sieve tubes embedded in tissues far surpassing them in mass has been extremely difficult to study. Attempts at restricting translocation studies to a small mass of tissue containing the sieve tubes were made by Mason and Maskell (17), but radioactive tracers offer a much easier solution to this problem. Several of these substances may be used simultaneously and with judicious placement and recovery it is possible to determine the path of movement and the metabolic participation of the mobile substances within undisturbed tissues.

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It was in the first use of P^{32} in phloem translocation studies that the mobile material was shown to be distributed logarithmically with respect to distance from the point of entry into the bark. This was for downward movement following application to a leaf (3). Subsequently, this distribution pattern was confirmed for P^{32}, S^{35} (2), C^{14}-labeled photosynthate (23), and simultaneously moving pairs of P^{32} with K^{42}, and Cs^{137} (21). This is the general pattern which would be expected of substances undergoing movement by diffusion, but Mason and Maskell (17) have shown that the velocity is far faster than could be accounted for by simple diffusion. In fact the diffusion constant for the mobile solute would need to be some 40,000 times greater than apparent values to account for the observed velocity. The wide occurrence of the logarithmic distribution pattern for substances undergoing translocation and the fact that this type of movement cannot represent a simple diffusion process made it desirable to investigate the significance of this pattern in relation to the mode of translocation.

The data upon which our knowledge of the velocity of transport in the sieve tubes is based comes from three types of experiments. These are: 1) calculations of downward velocity derived from the mass of material transported per unit of time through known cross sectional areas; 2) inferred velocities obtained from calculations based on increases in the concentration of translocates at specific distances from the assimilating tissues after definite time intervals, or by morphological responses of tissues at specific distances from the point of application of the substance producing the response; 3) the direct detection of a substance introduced at one place and recovered at another after a particular time interval. The following are examples of velocities, expressed in cm/hr, which serve to show the range of values which have been reported. Method 1: Dixon and Ball (11) 50, Crafts and Lorenz (8) 55 to 160. Method 2: Huber (13) 10 to 100, Day (10) 12 to 106. Method 3: Various virus particles (20) 8 to 150, Vernon and Aronoff (23) C^{14}-sucrose 84, Schumacher (19) fluorescein 16 to 65. It is of historical interest that the first calculation to be attempted, that of Dixon and Ball wherein 50 cm/hr was indicated resulted in a temporary rejection of the phloem tissue as the pathway of transport on the grounds that such a high velocity was incompatible with the structure of the tissue. Since Mason and Maskell (17) ruled out simple diffusion as a means of translocation in the phloem, there have been no successful attempts to link a certain velocity with a particular means of translocation.

If conclusive evidence could be obtained that different phloem mobile substances move at different velocities, this would be considered as evidence against the pressure flow mechanism—in particular if it is considered that the channel is completely inert toward the movement of solute. (The latter point is discussed below.) Vernon and Aronoff (23) claim some evidence of differential velocities for the simultaneous overall movement of glucose, fructose and sucrose, but they indicate that the difference may be accounted for in the diffusion step from the chlorenchyma to the phloem. Swanson and Whitney (21) indicate that their results may show differential velocities for two tracers moving simultaneously, but using their method of applying tracers, Koontz and Biddulph (14) find that the penetration time of their tracers to the phloem must have been in the order of an hour or more, thus making the rate of penetration to the phloem an even more critical factor than it might have been in the Vernon and Aronoff study. Horwitz (12) has analyzed the data of Swanson and Whitney, and while he disagrees with their analysis of the data, he finds a basis for their contention. The present investigation encompasses velocity studies wherein three tracers are moving simultaneously in the phloem of a single plant.

The data which are available at the present time apparently cannot be used to ascertain the concentration of the solution which moves in the phloem. A 10 % solution of assimilates has sometimes been used for illustrative purposes in velocity calculations (6, 8, 11). Clements (5) used values of 0.62 and 1.75 % which were obtained from phloem studies and which when converted to volumes of solution entering the fruits of the sausage tree gave values of 5.4 L and 1.76 L per day. Even had he used a value of 10 % the indicated volume of solution flowing into the fruits i.e., 0.35 L, would seem excessive. Crafts and Lorenz (8) subsequently reached the conclusion that phloem exudation could not be considered a manifestation of normal food movement by mass flow. Later Crafts (7) stated that all water moving in the phloem may be used by the receiving tissues for growth (except in storage tissues). One obvious conclusion to be drawn from the above studies is that it will be difficult to establish a limiting value for solvent volume as may fit a particular hypothesis of movement.

It does not seem likely that evidence based on either velocity, per se, or concentration of the translocate in the sieve tubes will be the critical factor in the selection of the mechanism for translocation. The best use for tritiated water then would be to test differential velocities and to establish the distribution pattern of this tracer in relation to those with which it is applied. Therefore, the initial objective of the present investigation was to provide quantitative data on the simultaneous translocation in the phloem of three widely differing substances. In addition contributions have been made to our knowledge of the metabolism of phosphate during its translocation.

Methods

The conditions necessary for a critical comparison of the translocation of the three tracers were simultaneous application to a leaf, and detection of individual radioisotopes in successive stem sections as they approached the root system. Experimental data were obtained on the rates of movement and the con-

centration patterns in the stem for each individual tracer.

While the data presented are for a relatively few plants, they are strengthened by the fact that the three substances moved simultaneously in the phloem tissues of a single plant. In addition, their behavior as individuals corresponded favorably to the observed average behavior of P^{32} in approximately 100 individuals, THO in 10, and C^{14}, administered either as labeled sucrose or as $C^{14}O_2$, in six individuals. It will become apparent later that the path of $C^{14}O_2$ through the photosynthetic mechanism to sucrose, which was then exported, proved no handicap as when applied with THO and P^{32} the C^{14} tracer was the first one to reach the root.

Red Kidney beans, grown to an age of 12 days from the time of the straightening of the hypocotyl in an aerated one-half strength Hoagland solution, with micronutrients, were used throughout the investigation. The environmental conditions during the growth and treatment periods were: temperature, $23 \pm 1°$ C; light, artificial fluorescent (2 daylight + 10 soft white tubes) on a twelve-hour photoperiod, 1000 to 1200 ft-c; relative humidity, 60 ± 5 %. The growth room was protected from "fall out" radioactivity by high efficiency filters—a precaution which was found to be necessary for accurate results. The $NaH_2P^*O_4$ in THO was sprayed directly on to the under surface of the terminal leaflet of the 1st (lowermost) trifoliate leaf. Application was to a circular area one inch in diameter, the center of which was median to the margins and 1 3/8" from the pulvinus. The C^*O_2, at a concentration slightly above 1 % by volume, was confined with 5 ml of air over the upper epidermis directly above the sprayed area. Amounts applied are given in the legends. Migration periods of 15, 20 and 30 minutes were allowed after which the stem below the node of the treated leaf was immediately cut into six 1-inch sections for analysis. The procedures for the analyses of the three individual tracers follow. The three tracers were applied as THO, $NaH_2P^{32}O_4$ (pH 4.0), and $C^{14}O_2$.

The one-inch sections were frozen for storage, then placed singly in a vacuum line for THO analysis (see below). After removal of the THO, the sections were subdivided and refluxed with 80 % ethanol in a soxhlet apparatus. The extracted radioactivity due to P^{32} and C^{14} was determined by the use of a 27.6 mg/cm^2 Al filter and an internal sample counter. The filter removed all but 0.09 % of the radiation from the C^{14} and required a correction factor of 1.48 to restore that part of the P^{32} radiation spectrum also absorbed. The total counts from P^{32} and C^{14} (unfiltered) minus the P^{32} (filtered) $\times$ 1.48 gave the counts due to C^{14}. The radioactivity (P^{32} and C^{14}) in the residue was determined similarly.

When tritiated water is used as a tracer, the contamination of the remainder of the plant with tritiated water vapor from the treated leaf must be avoided. To accomplish this the tritiated water was held under glass seals until dispensed in a glove box. It was transported to the plant treatment chamber after the plant had been properly placed therein. The stem of the plant below the node of the leaf to be treated, and through which the tritiated water was to be traced, was wrapped with two or more layers of saran plastic film. The treatment chamber, made of transparent plastic, contained three compartments; one for the roots in their container (A), an intervening compartment (B) to hold the remainder of the plant, and a compartment (C) for the leaflet which received the tracers. Effective gas seals were employed where the plant parts passed through the compartment dividers. The leaflet which received the tracers was further enclosed in a transparent plastic box of small volume into which the sprayer and the CO_2 reservoir were incorporated. The CO_2 reservoir was sealed to the leaf surface with silicone grease to retain the C^*O_2. There was no evidence of injury from silicone within a 60-minute period.

A small air conditioner serviced compartments B and C separately maintaining temperature and humidity conditions as above. Each compartment was separately exhausted, the air not recirculating. Compartment C was maintained at 2 mm H_2O less pressure than B, and B in turn at 2 mm H_2O less than ambient conditions. Moistened blotting papers containing 0.3 gm H_2O when nearly saturated were maintained in compartments A and B during runs as a check on contamination. With the above precautions no contamination occurred in compartment A and none, or very little, in B for up to 30-minute runs, but for 60-minute runs as many as 14 cpm in 0.3 gm H_2O in the blotting paper of compartment A and 350 in B were detected.

Plant sections were removed in sequence, the first cut being at the base of the stem (compartment A) which removed the root. The second cut which followed immediately was through the stem at the node of the treated leaf (compartment B). The stem with its wrapper was then sectioned and the sections slid from the wrapper into sample vials.

Procedure for Tritium Analysis

The procedure for the preparation and counting of tritium was similar to that described by Wolfgang and Libby (24). Plant samples containing tritium as water were treated in one of two ways, depending upon the size of the sample. Those containing 0.5 gm or less of water were placed in a standard taper test tube and connected to the reduction tube. Samples containing more than 0.5 gm of water were treated to separate the water by distillation under reduced pressure and the water caught in a trap cooled with solid CO_2 and methanol. The ice was allowed to melt and 0.3 ml of the water was then reduced to hydrogen gas for counting.

Reduction of the water was accomplished by passing the vapor over zinc dust at 400° C. The frozen sample, in its uncovered vial, was placed in a standard taper test tube, cooled in a dry ice and methanol mixture, and connected to the reduction tube which con-

tained a mixture of 5.0 gm of zinc-metal dust and 2 gm of asbestos fiber. The asbestos had been previously heated to 650° C for 8 hours to remove any water. The reduction tube was connected to the vacuum system and pumped continuously while the temperature of the reducing mixture was raised to 400° C at which time the vacuum system was closed and pumping stopped. The vacuum in the system was now sufficient to give hydrogen with good counting properties. The freezing mixture was removed from around the test tube containing the sample, and the sample was heated by means of a heat lamp to evaporate the water. The water vapor coming in contact with hot zinc was reduced and the hydrogen collected in a bulb of suitable size on the vacuum system. Heating of the sample was continued until all water had been reduced. The heat was then removed from the reduction tube and the sample tube and the hydrogen gas allowed to cool to room temperature. Figure 1 shows the design of the vacuum line.

Tritium was counted in a brass walled counter tube 20.5 inches long and $2\frac{3}{16}$ inches in diameter closed on the ends by lucite disks. The center wire was # 30 nichrome.

For counting, an argon-ethylene mixture of 77 % argon and 23 % ethylene was introduced into the counter tube to a pressure of 6.5 cm Hg, followed by a suitable amount of the hydrogen to be counted, usually a pressure of about 3 to 12 cm Hg, depending upon the activity of the sample. The gases were allowed to mix completely, after which the mixture was counted. Counting was done at about 80 volts above the threshold voltage which was 1500 to 2200 volts, depending upon the amount of hydrogen in the mixture. Each sample was counted at two different pressures of hydrogen. An average result was reported. The background count for the tubes described, using the same gas mixture except that tank hydrogen was used, was about 350 cpm inside of a 2-inch lead shield. Between counts the counter tubes and the system were washed three or four times with hydrogen gas and a background counted. There has been no evidence of contamination of the counter tubes, even from the most active samples.

Results

The data are presented as a series of curves wherein the logarithm of the concentration of tracer

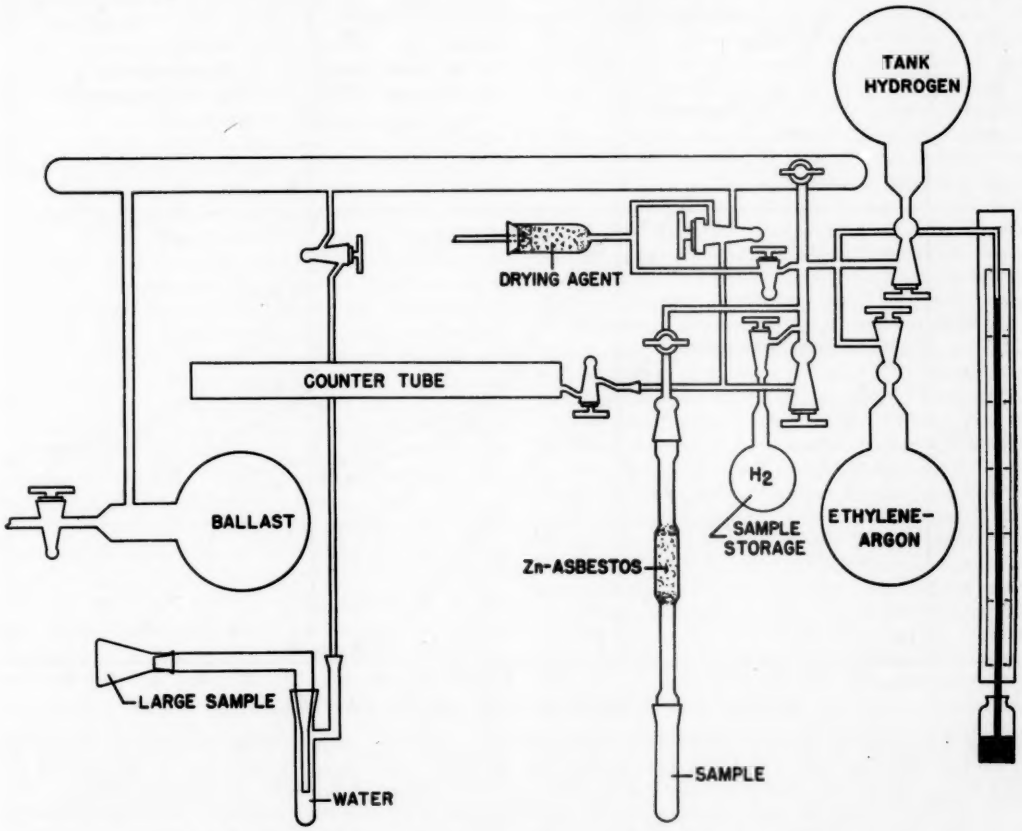


Fig. 1. The design of the vacuum system used for tritium analysis.

in each successive stem section is plotted against the distance the tracer had moved in a downward direction from the node of the treated leaf. The migration periods were 15, 20, and 30 minutes. These curves are shown in figure 2 A, B and C. All THO, C^{14} and P^{32} curves are plotted together in figure 2 D, E and F, respectively.

The velocity of each tracer was calculated from the total distance the tracers had moved, in a downward direction, from the point of application to the leaf epidermis during a 15-minute migration period. The data of figure 2 show the C^{14} tracer to have reached the root at the termination of this period. An extrapolated distance was not used in the calculations as the activity within the last section was near the limit of detection. The velocities expressed in cm/hr are as follows: P^{32} 86.5, THO 86.5, and C^{14} 107. There were some indications, gained from repetitions of these tests, and from the minute trace of P^{32} in the 6th section of the plant receiving a 20 minute migration period, that the P^{32} characteristically lagged slightly behind the THO, but this was not always evident. The necessity for the 1-inch sections, dictated by the amount of tissue necessary for the tritium analyses, made further refinement of velocity figures impossible. Only vigorous plants, free of nu-

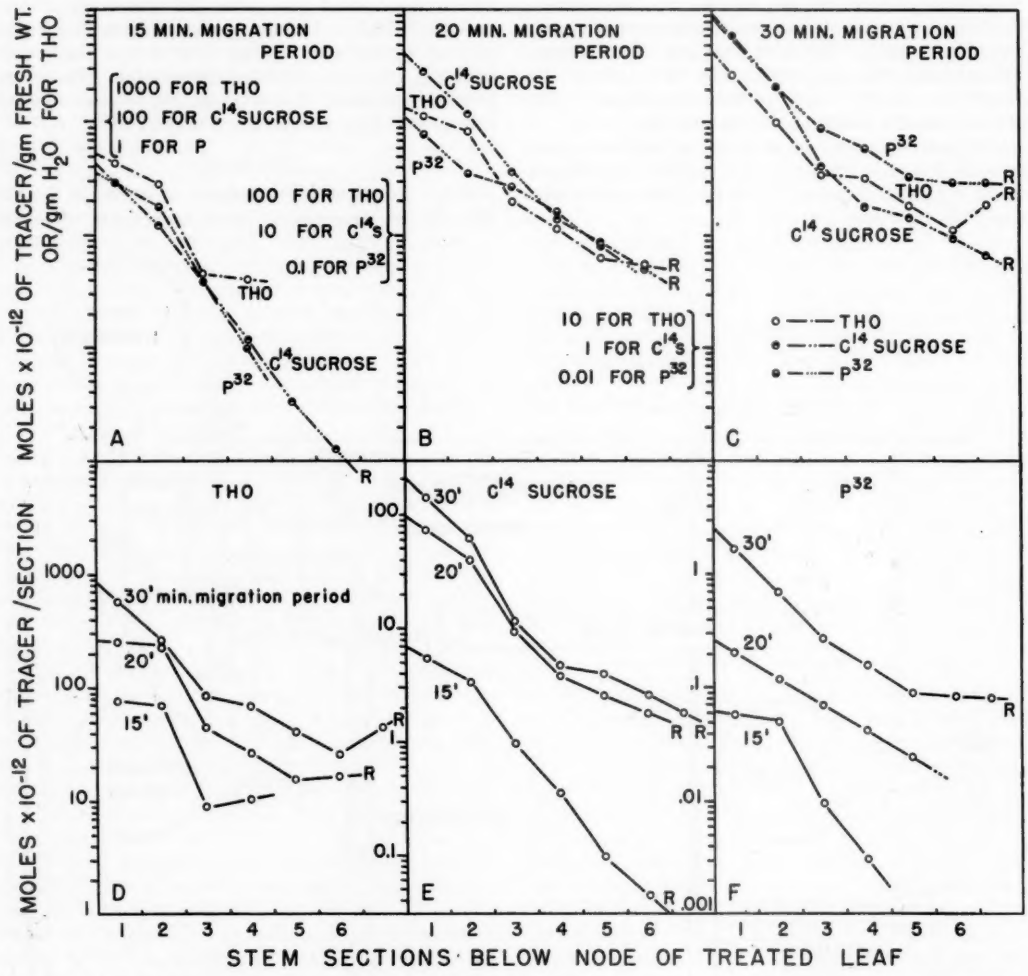


FIG. 2 A, B and C. The distribution of THO, P^{32} and C^{14} in the lower stem following simultaneous application to the 1st trifoliolate leaf. The 1-inch stem sections are numbered consecutively downward from the node of the treated leaf. R indicates that the tracer had entered the root within the duration of the migration period.

D, E, and F show the same data grouped by tracers instead of by migration periods. The dotted portion of the 20-min curve in F indicates only a trace of activity was detected in section 6. The amounts of tracer applied were: THO—2,500 μc, C^{14}—100 μc, P^{32}—322,166 and 160 μc for the 15-, 20- and 30-min periods, respectively.

trient faults and not subjected to undue manipulation, gave velocities of this value.

If the assumption is made that all three substances moved at the same velocity after reaching the phloem (106 cm/hr), but had different penetration times from epidermis to the phloem—the latter value is just less than 3 minutes (2.87) for P^{32} and THO. To this must be added the corresponding value for the penetration time for the C^{14} tracer, which includes diffusion of $C^{14}O_2$ to the plastids, its photosynthetic incorporation into sucrose, and the movement of sucrose from plastids to the phloem. However, in four trials where P^{32} and $C^{14}O_2$ were applied as above and allowed migration periods of 5 and 6 minutes, the detectable fronts of the P^{32} tracer, while yet in the petiole, were ahead of the C^{14} tracer in three trials and behind in one. This is strong evidence that the C^{14} tracer (as sucrose) had made the slower appearance in the phloem and its overall greater velocity was really due to its more rapid movement in the phloem of the stem.

The distribution patterns of the tracers in the lower stem are shown in figure 2. The curves for THO show the greatest departure from a linear relationship between the logarithm of the concentration and the distance of movement. The front of the THO stream was characterized by a relatively large amount of tracer, whether caught in the fourth section at the termination of the 15-minute period, in the sixth section at the termination of the 20-minute period, or in the 7th section after 30 minutes. This undoubtedly indicates that the application of the tracer water to the epidermis of the leaf accentuated the movement of liquid from this area, the local reduction in liquid tension at this spot facilitating the movement. If this were so, it would be expected that movement of water from leaves would be somewhat erratic as water tensions in the leaves were varied. The movement of tritiated water from treated leaves was maintained for periods of 60 minutes, however, with a significant decrease from a linear relation between the amount flowing and the migration time. The THO movement was expected to decline in time because of the influx into the leaf of unlabeled water from the root, thereby causing its dilution. Figure 3 A shows the time necessary for the attainment of a steady export rate.

An approximately linear relationship was found between the logarithms of the P^{32} and C^{14} concentrations in the stem and the distance of their movement for all migration periods. The inflections of the curves are successively higher for the longer migration periods. The slope of the 15 minute P^{32} curve was steeper than for longer periods. This could indicate a progressive increase in the entrance of P^{32} into the stem during the migration period. The stem section in which the node of the primary leaves fell usually had a disproportionately high activity which caused some of the irregularity in the curves, particularly if meristematic activity was pronounced at this node. This node usually fell in section 2, rarely in section 1.

The ratios of the total labeled sucrose to the total labeled water which was recovered in the stems showed that the values for the three migration periods of 15, 20, and 30 minutes were 3.4, 12.1 and 11.8 molal, respectively. To arrive at these values, the assumption was made that the sucrose was completely labeled with the tracer carbon confined above the leaf. This may not be justified for the 15-minute migration period where the labeled sucrose from the initial cycles of photosynthesis with $C^{14}O_2$ is just appearing in the stem (see fig 3 A), but for later cycling the assumption is justified since only a few percent of the CO_2 molecules available to the treated area of the leaf were not from the introduced sample. The low C^{14} sucrose value for the 15-minute period may be due to the above cause, and the low molal ratio, i.e., 3.4, to the high frontal concentration of THO.

Figure 3 A can be used for very rough calculations of the total amount of each tracer which would reach the root in a single day providing each square inch of mature leaf tissue were exporting at the maximum observed rates for the whole period. These rates are: 125, 28 and 0.28×10^{-12} moles/minute for THO, C^{14}-sucrose and P^{32}, respectively. Using 1000 minutes/day and an area factor of 100, the following results are obtained: THO = 0.24 μl, C^{14}-sucrose = 1 mg, P^{32} = 1 μgm. The time and area factors are both generous, however the rate factors are based on too few plants to be reliable beyond rough approximations.

If we assume the mass flow of solution at a concentration of 10 % sucrose, a figure frequently used (6, 8, 11)—10 μl instead of 0.24 μl of total water are required. To obtain this volume it is necessary to assume that there were 40 unlabeled water molecules flowing from the treated area for every labeled molecule, an assumption which does not seem too unreasonable, since there was no opportunity for the labeled water to come to equilibrium with the tissue water.

A comparison between the amounts of each tracer translocated, in relation to the amount applied to the leaf, is shown in figure 3 B. The C^{14} tracer shows the highest values with THO displaying the lowest.

The distribution of the P^{32} and C^{14} tracers, applied simultaneously, between the tissues interior and exterior to the cambium (conveniently referred to as wood and bark) was determined at the close of a 60-minute migration period. The wood contained 23.6 % of the total migratory P^{32} and 24.4 % of the total migratory C^{14}. The first two inches of stem below the node of the treated leaf were used for the analysis. This is consistent with calculations made on comparable data (60-minute migration period) for the cotton plant (3) wherein 25 % of the translocated P^{32} was present in the wood. In addition calculations showed that the percentage distribution between the two tissue systems remained relatively constant throughout the lower portion of the stem and indicated that the amount of loss from the sieve tubes was proportional to the concentration of tracer within them. At the close of a 20-minute migration period, the first one-inch section of wood below the node of

the treated leaf contained 31 % of the migratory THO present in that section of the stem. The next lower section of wood had 6 %, and in the third section there was none in the wood, while tritiated water was present in the bark. These data indicate that the lateral membranes of sieve-tube protoplasts are readily penetrated by sucrose, phosphate, and water and that really significant losses of these substances occur to the xylem. Loss from the sieve tubes was also evident in the P^{32} and S^{35} microautoradiographs in the studies of S. F. Biddulph (4).

The metabolic incorporation of the principal form of the mobile tracer into other constituents also constitutes an effective removal of tracer from the mobile fraction. Chromatographic methods were employed to determine the identity of the mobile substance and the extent of incorporation of this substance into others as movement occurred in the phloem. The tracer materials were removed from the tissue by extraction with 80 % ethanol in a soxhlet apparatus. This fraction contained approximately 95 % of the total C^{14} tracer and 44 % of the total P^{32}. This method has been widely used for the extraction of the phosphate esters.

Figure 4 shows the identity of the principal tracer containing substances (P^{32} and C^{14}) in the petiole of a treated leaf 60 minutes after application of the tracers. These substances were resolved on a filter

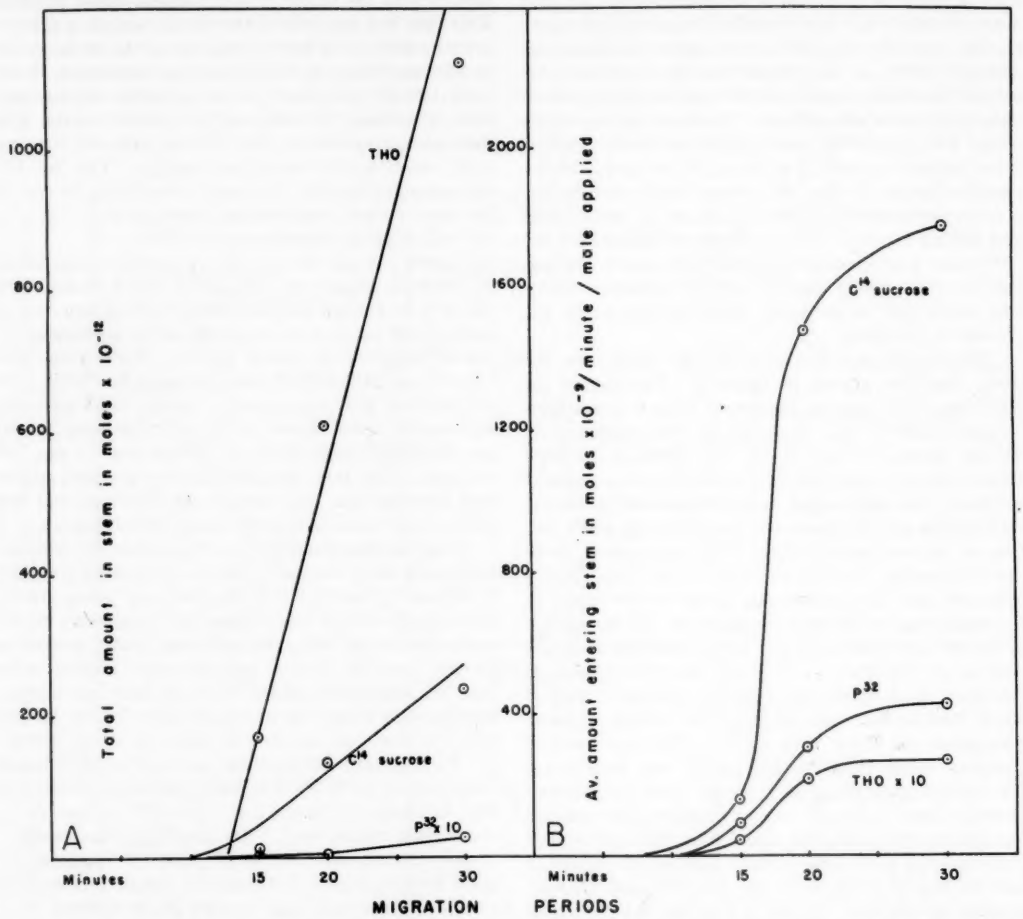


FIG. 3 A. The total amount of each tracer detected in the stems for the 15-, 20- and 30-min migration periods. The values for P^{32} have been multiplied by 10 to bring them onto the scale used to portray the other tracer values.

B. The amount of each tracer entering the stem in moles/min per mole applied. The amounts applied were: P^{32}, 85.5, 73.5 and 231×10^{-9} M, respectively for the 15-, 20- and 30-min migration periods; C^{14}, 4.46×10^{-6} M throughout; THO, 1.39×10^{-3} M throughout. The C^{14} data are expressed as moles sucrose/mole of C^{14} applied. There was always an excess of THO remaining on the surface of the leaf throughout the migration periods. The THO values have been multiplied by 10 to bring them onto the scale used to portray the other values.

paper chromatogram. This was then placed between two x-ray films with a 30.3 mg/cm² Al filter interposed between the chromatogram and one of the films in order to absorb the radiation from the C^{14}. The unfiltered film (A), which received both the P^{32} and C^{14} radiation, was removed after an exposure period of 9¼ days. The film behind the filter, which received that fraction of the P^{32} radiation energetic enough to penetrate the filter, was removed after an additional period of sufficient length to compensate for the absorption and the decay of P^{32} during the extended period. The total exposure time for this film (B) was 23¾ days. This film (B) shows only the P^{32} containing compounds. After the decay of the P^{32} radiation in the chromatogram, a third film (C) was exposed to record only the C^{14} containing compounds. This exposure was 9¾ days as in (A). No compensation was made for self-absorption in the filter paper (8.8 mg/cm²) or for the efficiency of exposure of the film by the radiation from the two isotopes.

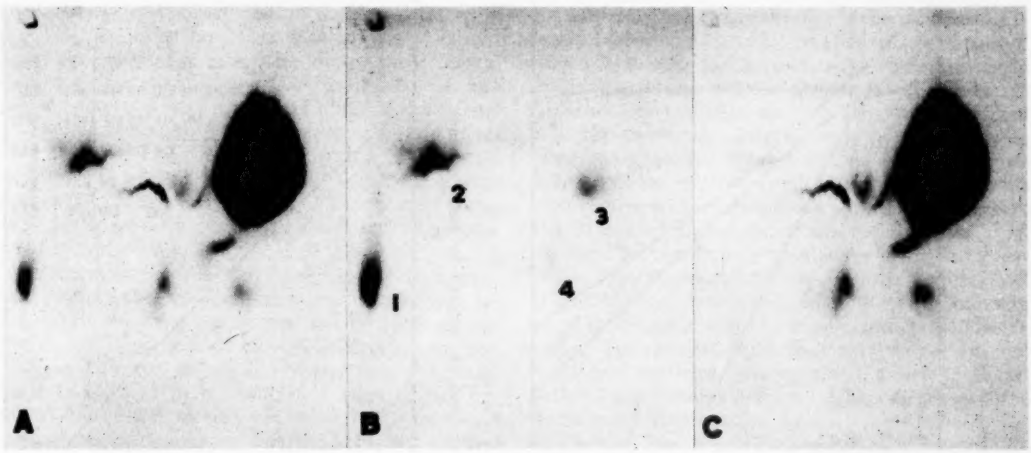


FIG. 4. Autoradiograms of a filter paper chromatogram showing the P^{32} and C^{14} radioactivity of substances present in the petiole 60 minutes after application of the tracers to a leaflet. A—the combined P^{32} and C^{14} radioactivity; B—the P^{32} containing compounds: 1—orthophosphate, 2—fructose, 1-6, diphosphate, 3—glucose, 6—phosphate, 4—glycerol phosphate (?); C—the C^{14} containing compounds. The major spot is preponderantly sucrose with fructose and glucose also present. The other substances were not identified. The origin is in the upper left corner. The solvents were: across, 1st methanol : NH_4OH : H_2O = 60 : 10 : 30, 15 hrs, 40 min; down, 2nd methanol : formic acid : H_2O = 80 : 15 : 5, 5 hrs, 5 min. Radioactivity applied to the leaf; C^{14} as C^*O_2—100 μc, P^{32} as $NaH_2P^*O_4$—180 μc in 50 μl.

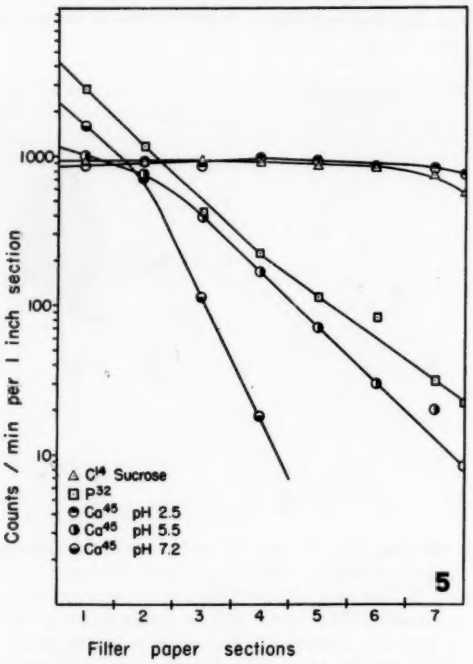
The C^{14} containing photosynthate which was translocated could not be resolved by the solvents used for the separation of the phosphate esters. Resolution of the C^{14} compounds was obtained by the procedure of Vernon and Aronoff (23). Sucrose was the principal metabolite exported by the leaf. Labeled glucose and fructose were also present, but the exact proportion of each was not determined. These results are consistent with those of Vernon and Aronoff obtained with soybeans (23).

The principal mobile form of the phosphate could not be determined with assurance. A number of phosphate esters, of which fructose, 1-6, diphosphate was predominant, were present as was inorganic phosphate. The fructose, 1-6, diphosphate was not significantly carbon labeled within the 60-minute migration period, indicating that its carbon chain was not of current photosynthetic origin. Phosphorylation undoubtedly occurred enroute. No information was obtained on the mobility of the fructose, 1-6, diphosphate or its retention within the sieve tubes. The mobility of this, or other compounds, should not be inferred solely on the basis of its presence in the extract, as tissue into which it could have moved was also extracted. Phosphoryl choline was not detected with the above methods or with those of Maizel et al (15).

An attempt was made to duplicate in vitro the essential features of the distribution pattern of P^{32} and C^{14}-sucrose in the bean stem. This was done by continuously adding a dilute tracer solution to the top of a previously wetted filter paper strip containing a pad of absorbent paper at the bottom to absorb the solvent (H_2O) and maintain the flow. These results are shown in figure 5, together with some results using Ca^{45} at three pH levels. The distribution patterns of P^{32} in the filter paper and in a bean stem are essentially similar. That is, there exists in both a linear relationship between the logarithm of the concentration and the distance from the point of application. C^{14} labeled sucrose, applied in water, was distributed rather uniformily along the strip as was Ca^{45} applied as $Ca^{45}Cl_2$ at a pH of 2.5. At pH 5.5 the Ca^{45} resembled P^{32} in its distribution pattern, but at pH 7.2,

the approximate pH of the phloem tissue, the Ca^{45} moved poorly—thus paralleling its behavior in the phloem.

Competition for solute, between the descending solvent and the adsorptive forces of the paper, produces the characteristic distribution patterns of the tracers in the filter paper strips. In the bean stem physical removal of tracer from the sieve-tubes and its metabolic incorporation into other compounds are known factors which operate to produce the observed distribution pattern of tracers in the stem. The part which adsorption might play as a factor contributing to the production of the logarithmic distribution pattern in the bean stem has not been determined.



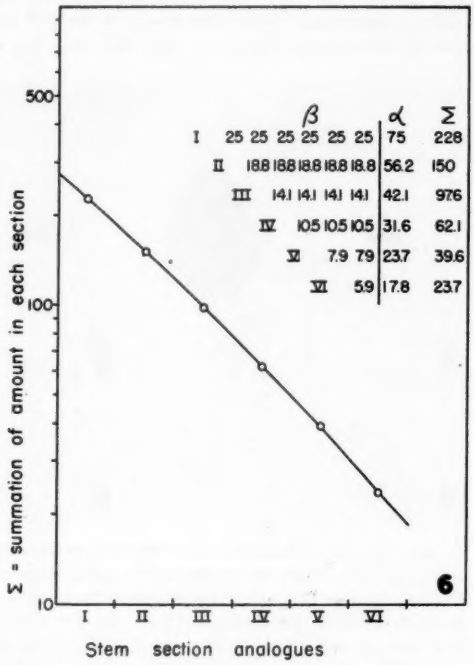


FIG. 5. A plot of the radioactivity in filter paper strips 2.2 cm wide and 25 cm long to which radioactive tracers were continuously applied at the top (section 1) and allowed to descend for 70 minutes. The phosphorus compound was $NaH_2P^{32}O_4$, the Ca was applied as $Ca^{45}Cl_2$.

FIG. 6. The theoretical distribution of a substance in a hypothetical stem when allowed to enter in successive aliquots one after another, flow downward stepwise from section to section, and move laterally at each level in amounts comprising 25 % of the total entering the section. Each successive aliquot entering the stem is stopped one section short of its predecessor. The amounts in each section are summarized and plotted to a log scale.

The following graphic analysis has been formulated to show a means whereby this particular distribution pattern might be produced, figure 6. The analogy is as follows: As the tracer enters the first section of the hypothetical stem, supposedly from a treated leaf, a constant proportion, e.g., 25 % of the total, is removed from the channel or otherwise lost from the mobile fraction, and the residue passed to the second section. If the same proportionate loss occurs here and the new residue passed to the third section, etc., and a new aliquot brought down behind the first, etc., there will result an approximately linear relationship between the logarithm of the concentration in the stem and the distance of movement. The same pattern will be present in the phloem and the xylem separately. The slope of the curve will be directly related to the amount of tracer which escapes from the mobile fraction in the sieve tubes, regardless of the mechanism of escape, and the slope of the curve will be steepened if the amount of tracer entering the stem increases during the experimental period. An additional requirement is that the loss of tracer from the mobile fraction be irreversible. The tracer is also visualized as remaining in the same plane at which it effected its escape from the sieve tubes. This would be true in plants for short migration periods, but for longer intervals some of the escaped tracer would enter the transpiration stream and be swept upward. A more precise mathematical treatment, with applications to the postulated mechanisms of transport, is presented by Horwitz (12).

DISCUSSION

The downward velocity of tritiated water, phosphate and sucrose in the phloem of the bean plant compares favorably with the upper limits of the val-

ues obtained by various other means. To find that the overall velocity of the C^{14} tracer, from the epidermis as $C^{14}O_2$ to the root as sucrose via the photosynthetic mechanism and phloem export, was greater than for the THO and P^{32} applied directly to the under surface of the leaf, strongly suggests a need for a closer investigation of the absolute velocities of different tracers in the phloem. The data of Vernon and Aronoff (23) and Swanson and Whitney (21) also suggesting independent velocities for simultaneously moving substances support this need.

The lack of similarity between the distribution patterns for THO, P^{32} and C^{14} is of considerable interest. It will be recalled that some C^{14}-labeled sucrose preceded the THO and that the P^{32} tracer accompanied or lagged behind it, yet neither the P^{32} nor the C^{14} tracer was distributed in the same pattern as the THO in the stem. This, together with the evidence for independent velocities for the three tracers, indicates that solvent movement cannot be the sole factor concerned with the distribution of the solute. The solute molecules definitely come under the influence of some factor or factors within the channel which are in addition to the effects which the solvent may have. These may be the diffusion step across sieve plates or metabolic forces associated with movement within sieve tubes.

Unfortunately, from the standpoint of discrimination between the possible mechanisms of transport, independent velocities per se need not be more damaging to one hypothesis of movement than to another. That is, however, unless in pressure flow the protoplast is assumed to be completely inert toward the movement of both solvent and solute. This may have been the interpretation of Crafts (6, 7), but the real essence of the pressure flow mechanism rests in the mode of solvent movement and pressure flow can be attacked only partially on the ground that the channel may influence the movement of the solute.

The criticisms against the pressure flow mechanism justifiably include its failure to deal with the effects which the protoplast may have on the movement of solutes. But even though we were to reject the pressure flow concept entirely, we cannot reject osmosis and whenever sieve tubes contain sucrose in solution and their longitudinal permeability exceeds their lateral permeability, there must be operative at least some rudimentary manifestations of the so-called pressure flow mechanism. The turgor of the sieve tubes is easily explained in this way. One might assume, as Van Overbeek (22) has done, that movement of solutes over protoplasmic surfaces (as in chromatography) must be given consideration, but if adsorptive forces were strong enough to overcome the competition which the solvent might have for the solute, thereby preventing the development of an osmotic pressure, the rate of movement of solute over these surfaces undoubtedly would be too slow to correspond with the observed velocities. We see no immediate need for abandoning the basic concept of pressure flow when it is applied to movement into the stem, via mature phloem, from a particular leaf.

One of the most disturbing features, for the well-being of the pressure flow concept, is the marked loss from the sieve tubes of both solute and solvent which occurs as the tracers enter the stem from a leaf. The rapidity with which tangential movement within the phloem of the stem occurs was shown by S. F. Biddulph (4); both the latter and the present study show an equally rapid radial loss to the xylem. The loss, at any level of the stem, was shown to be proportional to the concentration of tracer in the sieve tubes—so is markedly greater for that tissue immediately below the node at which entrance into the stem occurs.

Lateral loss of sugar from sieve tubes was described as early as 1928 by Mason and Maskell (17), but they concluded that the sieve tubes . . . "are to a considerable extent isolated from the outer tissues of the bark, so that the head of sugar concentration in the sieve tubes is only slightly dampened by leakage into the surrounding tissues." They further stated that the movement longitudinally along the sieve tubes is enormously greater than laterally to parenchyma, yet the great area of lateral contact makes the total movement to parenchyma appreciable. Maskell and Mason (16) also found the radial spread from the sieve tubes relatively greater for nitrogenous materials than for carbohydrates. Mason and Maskell (17) considered lateral loss from the sieve tubes to occur as though it could be accounted for by diffusion alone. The present results show a very extensive and rapid loss, as though there existed a well-developed pathway for solute movement. This, of course, is the traditional function of the rays.

Currier, Esau and Cheadle (9) have recently reexamined the matter of permeability of the sieve tube protoplasts and confirmed the evidence that they are normally plasmolyzable with sucrose. This would normally indicate a low permeability to sucrose of both the longitudinal and lateral sieve element membranes. But since sucrose moves freely through a linear series of sieve elements in an intact plant, the conclusion that sieve element membranes are impermeable, longitudinally, to sucrose is thereby denied. The observations of Currier et al (9) perhaps then confirm Rouschal's (18) suggestion that plugging of the sieve plate is necessary for the induction of plasmolysis. In any event, to produce plasmolysis with sucrose the sieve tube must somehow be denied its normal demonstrated function of the longitudinal passage of sucrose. The rapid lateral loss herein demonstrated at least raises some questions concerning the degree of impermeability of the lateral membranes.

The lateral loss of P^{32} from sieve tubes of the bean confirms the earlier similar observations of Biddulph and Markle (3) for cotton. The data for C^{14}-sucrose and THO extend these observations and the results of S. F. Biddulph (4) add S^{35} to the list of substances which are transferred laterally from sieve tubes to other tissues including the xylem—as these substances move downward in the stem. The ultimate fate of a

fraction of the material which leaves the phloem is upward movement in the xylem to maintain the circulation of those substances not metabolically captured —as described earlier (1). It is this lateral loss which is responsible for the production of the logarithmic distribution pattern described above. Therefore, this distribution pattern, being in part the result of the lateral loss of solute from the sieve tubes, cannot be used as evidence for a diffusion type of movement.

Because we have evidence of THO movement in the phloem, though none for the nature of the cause producing it, our present interpretation is that we cannot refute pressure flow as a mechanism, but our evidence may make it desirable to re-examine the proposed scope of its operation and emphasize the concept that the channel through which movement takes place is not inert to the passage of solutes. It is relatively easy to show the suitability of pressure flow to the delivery of solutes from the leaf into the stem, and perhaps for a limited amount of downward movement in fully mature sieve tubes, but it becomes difficult to apply this concept to all aspects of movement in the phloem. This latter point cannot be discussed until the data on upward movement in the phloem is presented.

Summary

THO, $H_2P^{32}O_4^-$ and $C^{14}O_2$ were applied simultaneously to the surface of a bean leaf and assayed individually in sections of the lower stem after 15, 20 and 30 minutes. The amount moving per unit applied was greatest for C^{14} and least for THO. The C^{14} moved largely as sucrose; the P^{32} as phosphate or fructose, 1-6, diphosphate, not containing the C^{14} label. The velocities for P^{32} and THO was 87 cm/hr and for C^{14}, 107 cm/hr including time for movement to the phloem.

The estimated total downward export per plant per day for C^{14}-sucrose, THO and P^{32} was 1.0 mg, 0.24 μl, and 1.0 μgm, respectively. The ratio of C^{14} sucrose to THO was approximately 12 molal but an undetermined amount of unlabeled tissue water was undoubtedly also translocated. There was an approximately linear relationship between the logarithm of the P^{32} and C^{14} concentration and the distance from the point of entry into the stem. THO departed from this pattern, which is evidence that some features within the sieve tubes may restrict the freedom of solute molecules to accompany the solvent. A loss from the sieve tubes to the xylem amounting to approximately 24 % for P^{32} and C^{14} and 31 % for THO indicated a marked permeability of the lateral sieve tube membranes. The logarithmic relationship between tracer concentration and distance was explained on the basis of loss of tracer from the sieve tubes.

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ELECTRON TRANSPORT AND CYTOCHROMES OF SUB-CELLULAR PARTICLES FROM CAULIFLOWER BUDS[1]

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The fact that substrates of the tricarboxylic acid cycle and reduced diphosphopyridine nucleotide (DPNH), one of the coenzymes involved in the oxidation of these substrates, are oxidized by particulate structures of plant cells is well established (1, 3, 8, 10, 13, 14, 18, 20). Laties (15) has shown that mitochondrial particles from cauliflower oxidize α-ketoglutarate, malate, pyruvate, succinate and oxalacetate with accompanying phosphorylation. The purpose of this paper is to determine the type of particles in a homogenate of cauliflower buds, examine some of the components of these particles, and determine their enzymatic characteristics with respect to the oxidation of DPNH and succinate.

In order to localize the particles which oxidize DPNH and succinate an homogenate of cauliflower tissue was separated into several particulate fractions by differential centrifugation. Each of the fractions was washed thoroughly by repeated sedimentation in fresh sucrose and any obvious contamination by lighter or heavier fractions removed.

MATERIALS AND METHODS

PREPARATION OF PARTICULATE FRACTIONS AND DESCRIPTION OF THE PARTICLES: Preparation of the particles was based on the procedure originally described by Laties (15). Homogenization was carried out in a Waring blendor operated at two-thirds full speed for 20 seconds on batches of 80 gm of cauliflower buds suspended in 100 ml of 0.5 M sucrose containing 0.001 M sodium versenate at pH 7.0. All operations were at 0 to 4° C. This procedure was found to yield particles with DPNH and succinoxidase activities equal to those of particles isolated from a homogenate prepared by grinding in a mortar and pestle with fine sand. Addition of 0.001 M versene was found to stabilize the activity of the particles for storage at – 20° C. The pH of the homogenate varied from 6.3 to 6.6.

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The use of 0.05 M phosphate buffer or adjustment of the pH of the final suspension to pH 7.0 by addition of KOH during the grinding was found to produce less active particles, and to cause difficulty in the separation of fractions. The homogenate obtained from the blendor was squeezed through three layers of cheesecloth to separate most of the cell debris and subjected to a series of centrifugations at increasing velocity after removal of the pellet at each stage. The pellets were resuspended in 0.5 M sucrose and 0.001 M versene in a Potter-Elvehjem homogenizer. Starch granules were left on the bottom of the tube. The centrifugation was in the following order: three minutes at one-half speed in the one-liter angle head of the International PR-1, three minutes at full speed in the PR-1, 10 minutes at 8,000 rpm in the number 30 head of the Spinco model L, 10 minutes at 15,000 rpm in the Spinco, 15 minutes at 40,000 rpm in the number 40 head of the Spinco and 1 hour at 40,000 rpm in the same head. The final supernate was clear with a slight fatty layer on the surface.

Table I shows a list of the fractions with the approximate centrifugal force and time of centrifugation, a description of their appearance, and the designation under which they will be discussed.

TABLE I

CONDITIONS FOR SEPARATION OF PARTICLES

FRACTION	CENTRIFUGAL FORCE × G	TIME OF CENTRIFUGATION, MIN	DESCRIPTION OF PELLET	DESIGNATION
1	600	3	Sticky green grey	Cell debris
2	2,000	3	Fibrous grey	Fibers
3	5,000	10	Smooth brown yellow	Medium
4	25,000	10	Smooth pale yellow	Medium light
5a	105,000	15	Smooth pale yellow	Packed light
5b	105,000	60	Translucent bright yellow	Fluffy light

ASSAY OF ENZYMATIC ACTIVITY: DPNH oxidase activity was determined spectrophotometrically by following the oxidation of DPNH by decrease in optical density at 340 mμ after addition of enzyme or DPNH to buffer mixtures described below. The buffer solutions and cell compartment of the spectrophotometer were maintained at 37° C. The reaction was started by addition of 0.01–0.05 mg enzyme protein or 0.04 ml of 0.2 % DPNH (reduced diphosphopyridine nucleotide) to the reaction mixture contained in 1-ml cuvettes with 1 cm optical path length. The total volume was one ml. No oxidation occurred in the presence of 3×10^{-5} M KCN, 0.02 μgm of antimycin A (22), 0.1 μgm 2,heptyl,4,hydroxyquinoline-N-oxide (4), or in the case of the medium particles under anaerobic conditions after flushing the solutions with helium. The influence of cytochrome c was determined by adding 0.01 ml of a 1 % solution of Sigma cytochrome c to the reaction mixture. 40 micromoles TRIS [tris(hydroxymethyl)aminomethane] chloride at pH 7.4 and potassium phosphate (40 micromoles) at pH 7.4 were used as buffers in routine assay procedures. At this pH in TRIS buffer maximum oxidation rate was achieved without addition of cytochrome c, whereas in phosphate buffer maximum rate was achieved only by addition of cytochrome c. The requirement for cytochrome c was directly dependent on phosphate concentration in the assay medium, but at higher levels of phosphate addition of cytochrome c did not completely restore activity. The affect of phosphate concentration of DPNH oxidase is shown in table II, and the effect of pH in the two buffer systems is shown in figure 1. The standard assay used in later work was based on these relationships. The extinction coefficient of DPNH was taken as $6.22 \times 10^6 \times cm^2 \times mole^{-1}$.

TABLE II

EFFECT OF PHOSPHATE CONCENTRATION ON DPNH OXIDASE ACTIVITY OF MEDIUM PARTICLE

PHOSPHATE, MICROMOLES	DPNH OXIDASE RATE *	
	NO CYT. C	CYT. C ADDED
None **	0.095	0.097
20	0.080	0.085
40	0.042	0.080
80	0.020	0.048

* Assayed at 37° C and pH 7.4 with or without the addition of 0.1 mg cytochrome c in a total volume of 1.0 ml. Specific activity in micromoles DPNH/min × mg.

** Forty micromoles TRIS chloride pH 7.4 as buffer.

The rate of oxidation of DPNH by cytochrome c (DPNH–cytochrome c reductase) was determined by following reduction of cytochrome c by increase in absorbance at 550 mμ at 37° C in a reaction mixture which contained 100 micromoles potassium phosphate pH 7.4, 0.6 ml of 0.2 % DPNH, 0.1 ml of 1 % cyto-

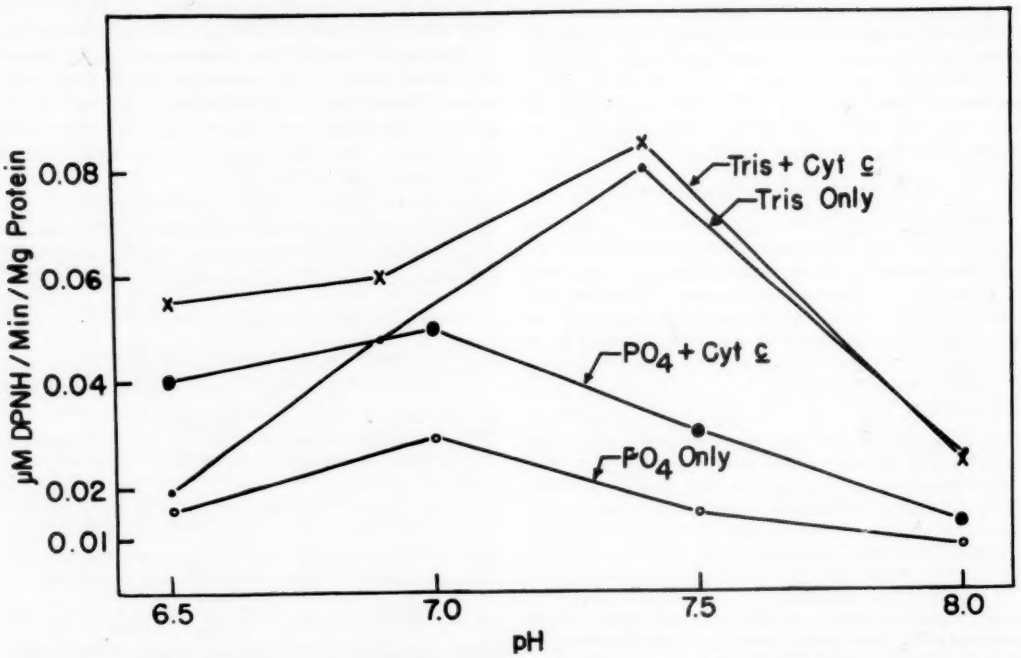

FIG. 1. The effect of pH and buffer on the requirement for cytochrome c in the DPNH oxidase activity of the medium particle. Assayed with 40 micromoles TRIS chloride or 80 micromoles of potassium phosphate buffer with 0.1 mg cytochrome c in a total volume of 1.0 ml.

chrome c and 0.3 micromoles potassium cyanide in a total volume of 1.0 ml. The oxidation rate was the same when phosphate was replaced by TRIS chloride at the same concentration and at the same pH. The difference between the extinction coefficient for oxidized and reduced cytochrome c was taken as $19.7 \times 10^6 \times cm^2 \times mole^{-1}$.

Diaphorase activity was determined at 37° C by following the reduction of 2,6-dichlorophenolindophenol by decrease in absorbance at 600 mμ after addition of enzyme or DPNH to a reaction mixture containing the same components as were used in the cytochrome c reductase assay, except that 0.1 ml of 0.2 % indophenol was substituted for cytochrome c. In both the diaphorase and cytochrome c reductase assays there is considerable reduction of indophenol and cytochrome c by the homogenate and supernate without addition of DPNH. All rates given are corrected for this endogenous rate. The extinction coefficient for indophenol was determined to be 16.1×10^6 $cm^2 \times mole^{-1}$ at this pH.

Succinic oxidase (5), succinic cytochrome c reductase (9) and cytochrome oxidase (16) were determined as previously described.

Protein was determined by biuret reaction in presence of deoxycholate (9). Beef serum albumin (Armour and Co.) was dialyzed 25 hours against distilled water before use. Absorption spectra were recorded on the Beckman DK-1 spectrophotometer. Enzymatically reduced DPNH and cytochrome c were obtained from Sigma Chemical Co., St. Louis and antimycin A from Wisconsin Alumni Research Foundation, Madison.

TABLE III

ENZYMATIC ACTIVITY OF PARTICLES

FRACTION	SPECIFIC ACTIVITY *				
	DPNH OXIDASE	DPNH CYT. C REDUCTASE		DIAPHORASE	CYTOCHROME OXIDASE
		NO ANTIMYCIN	ADDED ANTIMYCIN		
Orig. homogenate	0.028	0.008	0.008	0.029	0.070
Cell debris (1)	0.003	0.001	....	0.016	0.019
Fibrous (2)	0.060	0.014	0.019	0.072	0.042
Medium (3)	0.137	0.040	0.025	0.156	0.250
Medium light (4)	0.063	0.039	0.030	0.102	0.070
Light (5a)	0.033	0.035	0.050	0.081	0.033
Light (5b)	0.015	0.050	0.055	0.052	0.007
Final supernate	0.014	0.003	0.003	0.006	0.002

* All rates are expressed as μeq/min × mg protein for a two-electron transfer. Thus the observed rate of oxidation of reduced cytochrome c in micromoles is two times that given here. DPNH oxidase assayed in TRIS buffer.

TABLE IV

RECOVERY OF ENZYMATIC ACTIVITY IN THE PARTICULATE FRACTIONS

FRACTION	UNITS OF ACTIVITY **					
	PROTEIN, MG	DPNH OXIDASE	DPNH CYT. C REDUCTASE		DIAPHORASE	CYT. OXIDASE
			NO ANTIMYCIN	ADDED ANTIMYCIN		
Orig. homogenate*	4560	128.0	45.0	45.0	129.0	320
Cell debris (1)	69	0.2	0.1	....	1.1	1
Fibrous (2)	56	3.4	0.8	1.3	3.2	2
Medium (3)	307	42.2	12.3	7.7	48.0	77
Medium light (4)	124	7.8	4.9	3.7	12.7	9
Light (5a)	112	3.7	3.9	5.6	9.1	4
Light (5b)	81	1.2	4.1	4.4	4.2	1
Final supernate	3460	49.0	10.4	10.4	21.0	7
Recovery	4189	107.5	36.5	33.1	99.3	101

* The original homogenate represents the filtered juice from 540 gm wet wt of cauliflower buds.

** Units of activity refers to μeq of DPNH which would be oxidized per minute by the total fraction and represents the specific activity and the total protein in each fraction.

RESULTS

DISTRIBUTION OF ENZYMATIC ACTIVITIES: The specific activities of each of the particles for oxidation of DPNH by oxygen, cytochrome c and indophenol and for oxidation of reduced cytochrome c by molecular oxygen are given in table III as well as some data on the antimycin sensitivity of the cytochrome c reductase reaction. DPNH oxidase activity is concentrated in the medium particles, whereas the DPNH cytochrome c reductase activity is relatively high in the lighter particles and is not inhibited by antimycin A, except for a small inhibition in the medium particles. The diaphorase activity is in each case closely approximate to a summation of the DPNH oxidase and DPNH cytochrome c reductase activities. The ability to oxidize reduced cytochrome c by molecular oxygen is concentrated almost exclusively in the medium particle, and in other particles is present in proportion to the DPNH oxidase activity except in the cell debris where the activity, although low, is five times the DPNH oxidase activity. Since this cell debris material presents many technical problems a further investigation of the nature of this cytochrome oxidase activity has not been attempted at the present, although this activity could be of considerable significance in view of the large mass of the cell debris fraction which is eliminated from the fractionation by the preliminary filtration step.

In order to determine that the isolated particles account for the activities in the original filtrate the

TABLE V

SUCCINATE OXIDATION BY PARTICULATE FRACTIONS *

FRACTION	SUCCINOXIDASE		SUCCINIC CYT. C REDUCTASE
	NO CYT. C	ADDED CYT. C	
Fibrous (2)	0.006	0.010	0.004
Medium (3)	0.008	0.015	0.006
Medium light (4)	0.006	0.010	0.005
Light (5a)	0.001	0.002	0.002

* Activity expressed as micromoles succinate oxidized per minute per mg protein.

recovery of protein and enzymatic units has been determined (cf. table IV). The recovery in each instance is good in view of the losses which can be expected from the several handlings except for unexplained loss of cytochrome oxidase.

The DPNH oxidase activity of each particle has also been determined in the presence of an equivalent amount of supernate, and the light and medium particles have been mixed in equal proportions for assay, with no evidence of mutual stimulation or inhibition.

OXIDATION OF SUCCINATE: The ability to catalyze the oxidation of succinate by oxygen or cytochrome c is restricted primarily to the medium particles. The succinoxidase rate is stimulated about twofold by addition of cytochrome c. This effect may be related to the use of phosphate buffer in the assay. No attempt has been made to introduce tris buffer into the assay. The rates of succinate oxidation by the particles are shown in table V. It has not been possible to get sufficiently accurate rates on the original homogenate because of a large endogenous rate which renders the balance of units somewhat untrustworthy.

CYTOCHROME COMPONENTS: The presence of cytochromes has been demonstrated in all particulate units of animal and plant cells involved in electron transport which have been examined in this respect. In the case of animal mitochondria it has been possible to clarify the suspension by the use of deoxycholate and obtain spectra showing the absolute absorbance of these cytochromes. When cauliflower particles are treated in this manner the suspension is partially clarified, but the spectrum still shows some end absorption from turbidity with large peaks at 475 and 450 mμ which appear to represent carotenes in the plant particles. After addition of dithionite to this suspension two slight shoulders appear in the region of 600 and 560 mμ. It is of interest that there is no peak at 665 mμ in any of the particles, except for the cell debris which may indicate the presence of a small amount of chlorophyll in this material. It has been found, however, that excellent spectra of the cytochromes can be obtained if the spectrum of a clarified suspension reduced by dithionite is measured against a suspension which has not been reduced, as a reference, so that the spectrum obtained represents the reduced – oxidized difference spectrum. Difference spectra of the particles obtained in this manner are of two general categories. The first type is found in the heavier particles, whereas, the second is found in the lighter material. The medium particles show peaks on reduction at 603, 525 and 428, with a broader band from 560 to 550 mμ, a small shoulder around 440 mμ and a drastic decrease in absorption in the region around 400 mμ. The light particles show peaks at 559, 525 and 428 mμ with very little absorption at 603 mμ, and do not show the drastic decrease in absorption at 400 mμ which is characteristic of the heavy material. These spectra are shown in figures 2 and 3.

STABILITY: The DPNH and succinoxidase activities of these particles decline only slightly when the particles are stored in 0.5 M sucrose containing 0.001 M versene at – 20° C for two or three days. After seven days of storage there is a considerable decline in DPNH oxidase activity when assayed either in TRIS chloride buffer or in phosphate buffer in the presence of cytochrome c. A good part of this loss in activity can be restored by the addition of 0.1 ml of 10 % solution of dialyzed beef serum albumin to the assay mixture. In many cases a complete restoration of activity has been achieved. On continued storage there is a further slow irreversible decline in activity under all assay conditions which have been tested. The DPNH oxidase activity of fresh and seven-day-old material is shown in table VI.

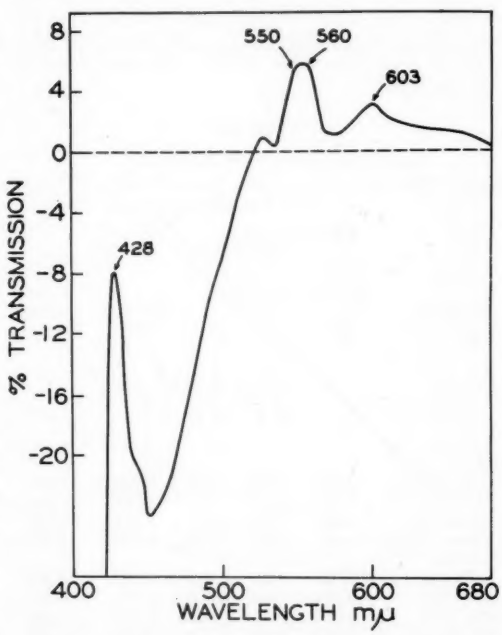

FIG. 2. Reduced – oxidized difference spectrum of the medium particle. Three and seven-tenths mg protein, 0.01 mg potassium deoxycholate and 40 micromoles potassium phosphate in a total volume of 1.0 ml reduced with a few grains dithionite. One cm light path.

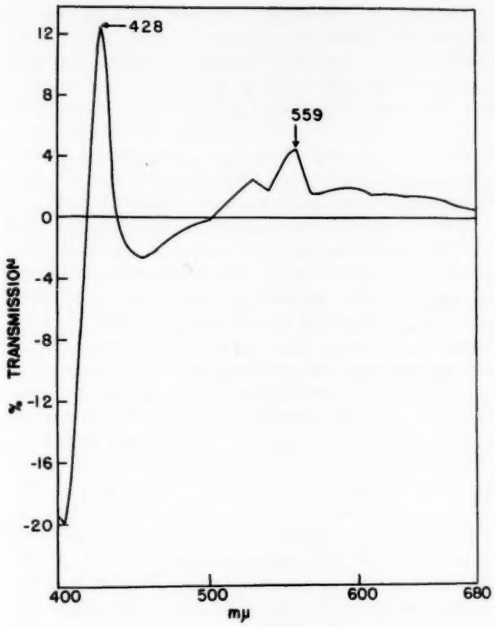

FIG. 3. Reduced – oxidized difference spectrum of the fluffy light particle. Three and one-half mg protein under the conditions described for figure 2.

The loss of DPNH cytochrome c reductase is much less than that of the DPNH oxidase, with 50 % of the original activity present after two to three weeks of storage. Beef serum albumin does not restore reductase activity under the assay conditions described.

OPENING EFFECT: Exposure of the electron transport particle isolated from beef heart mitochondria to deoxycholate has been shown to produce a requirement for cytochrome c in the oxidation of DPNH and succinate, with the corresponding appearance of DPNH and succinate cytochrome c reductase activity (6, 16). Treatment of the medium cauliflower particles with deoxycholate under the same conditions causes complete loss of DPNH oxidase activity which cannot be restored by addition of cytochrome c, as well as almost complete loss of cytochrome c reductase activity. An effect which is somewhat similar to this opening phenomenon has been observed, however, when the cauliflower medium particles are exposed to 0.1 M phosphate in the presence of 10 % ethanol. A suspension of medium particles in 0.5 M sucrose was mixed with an equal volume of 0.2 M potassium phosphate and ethanol was added slowly to a final concentration of 10 %. The suspension was centrifuged for 15 minutes at 105,000 × g in the No. 40 Spinco head. The pellet was washed once by resuspending in 0.5 M sucrose followed by centrifugation and finally taken up in 0.5 M sucrose.

The treated particles show twice as much DPNH and succinic cytochrome c reductase activity as the original particles. This increase represents new enzymatic units brought into action by the treatments employed and is not a purification of the pre-existing activity. In respect to cytochrome c reductase activity this phenomenon corresponds to the "opening" of beef heart ETP. The results differ however in that there is no proportional decrease in DPNH oxidase units, or corresponding increase in reduced cytochrome oxidase units. It also differs in that there is no increased requirement for cytochrome c in the DPNH oxidase activity of the treated particles either in TRIS or phosphate buffer. A comparison of the units of activity in the original and treated particles is shown in table VII. The additional DPNH cytochrome c reductase activity produced by the phosphate-ethanol treatment is inhibited at least 50 %, but never more than 80 % by 0.02 μgm antimycin A in all preparations tested.

TABLE VI

EFFECT OF AGEING ON DPNH OXIDASE ACTIVITY UNDER VARIOUS ASSAY CONDITIONS *

AGE OF PREPARATION, DAYS	BUFFER	DPNH OXIDASE SPECIFIC ACTIVITY			
		WITHOUT BSA **		WITH BSA **	
		NO CYT. C	CYT. C ADDED	NO CYT. C	CYT. C ADDED
1	TRIS	0.137	0.130	0.125	0.130
1	Phosphate	0.018	0.120	0.016	0.130
7	TRIS	0.019	0.022	0.056	0.056
7	Phosphate	0.014	0.028	0.014	0.056

* Particles were stored under conditions described in the text.
** BSA indicates beef serum albumin.

TABLE VII

EFFECT OF PHOSPHATE-ETHANOL TREATMENT ON ENZYME ACTIVITY OF MEDIUM PARTICLES

FRACTION	UNITS OF ACTIVITY *					
	PROTEIN, MG	DPNH OXIDASE		CYTOCHROME OXIDASE	DPNH-CYT. C REDUCTASE	SUCCINIC-CYT. C REDUCTASE
		NO CYT. C	CYT. C ADDED			
Original	66.0	3.0	11.1	4.6	6.8	0.9
Treated	44.4	2.1	7.5	3.9	10.0	2.5
Supernate	10.2	0.0	0.0	0.0	0.0	0.0

* Units of activity as defined in table IV. DPNH oxidase assayed in phosphate buffer.

DISCUSSION

On the basis of physical appearance there are four predominant particle types in the filtrate from a cauliflower bud homogenate as well as a large amount of soluble protein. The heavy fibrous material may be cell wall debris with a certain amount of lighter particles attached to it or enmeshed in it. The medium

particle seems to correspond to mitochondria on the basis of its enzymatic properties and the lighter fractions correspond to microsomes. The striking difference in physical appearance between the two light fractions should be noted, although they do not differ greatly in the enzymatic properties studied.

The medium particle is capable of catalyzing the oxidation of DPNH and succinate by molecular oxygen. Oxidation of DPNH is sensitive to antimycin A, 2,heptyl,4,hydroxyquinoline-N-oxide and cyanide. It is also inhibited by phosphate ion, and activity is restored by addition of cytochrome c. This particle resembles the heavy fraction of beef heart mitochondria, and would appear to contain very little of the phosphate stimulated DPNH oxidase system of the type found in the beef heart (5) or Azotobacter (2) electron transport particles (ETP). The inhibition by phosphate ion can best be explained at present on the basis that fairly concentrated salt solutions may extract cytochrome c from the particle, as has been shown with animal mitochondria (24), and a high exogenous level of cytochrome c causes replacement of this compound. Such a requirement for cytochrome c in the overall oxidation process does not necessarily mean that DPNH cytochrome c reductase activity can be shown in the particle, as is suggested by the fact that the reductase activity is not effected by assay in phosphate buffer, and from studies with deoxycholate treatment of beef heart ETP (6). We feel that the antimycin insensitive DPNH cytochrome c reductase of these particles must be caused by contaminating microsomes, or by an altered DPNH oxidase system indicated by the opening effect, since all of the complete DPNH oxidase activity is antimycin sensitive. This differs from Martin and Morton's interpretation of similar activity found in particles from silver beet (18).

The lighter particles contain an antimycin insensitive DPNH cytochrome c reductase and very little cytochrome oxidase activity and are thus similar to microsomal particles found in liver. Similar particles have been reported in silver beet by Martin and Morton (18) although the ratio of diaphorase to cytochrome c reductase in these particles is much higher under different assay conditions.

There is also a small amount of DPNH oxidizing capacity in the original homogenate which is not accounted for in the diaphorase activity, and is recovered in the final supernate. Such an effect could be expected since reducible substrates and the enzymes to carry out their reduction by DPNH could be expected in the homogenate (17, 1a, 21a). A similar soluble DPNH oxidase of unknown nature has been reported by Romberger (23) in barley root homogenates. On the other hand the diaphorase activity of the fractions is associated almost exclusively with the particulate components and is proportional to the DPNH oxidase or cytochrome c reductase activities. Therefore there is no evidence in this tissue for DPNH diaphorase which is not associated with the particulate enzymes.

The loss of cytochrome oxidase units during the fractionation cannot be explained at this time. A recombination of various fractions would be desirable in approaching this problem since some system other than the usual cytochrome oxidase may be operating here. For example a cytochrome peroxidase separated from a supply of H_2O_2, or polyphenol oxidase separated from phenolic substrates by the fractionation procedure could account for the lost activity.

The cytochrome components of the particles show the differences which would be expected between mitochondria and microsomes. The medium particles contain peaks at 603, 560 and 550 mμ which would indicate the presence of cytochromes similar to cytochrome a, b_3 and c or c_1 (11). The light particles, however, contain one predominant cytochrome which would appear to correspond most closely to cytochrome b_3. It is of interest that the 560 peak in the mitochondria is very close to the 559 peak in the microsomes so there is not evidence as yet for the existence of a separate b heme in the two particles. This 559 mμ cytochrome is also different from any reported in animal material and is apparently very similar to the b_3 of Hill and Scarisbrick (12) or Martin and Morton (19) with peaks reported at 560 and 425.

The nature of the component in the medium particles responsible for the decrease in extinction around 400 mμ is unknown. It cannot be accounted for on the basis of spectral properties of known hemes and flavins.

The ability of material like serum albumin to restore activity indicates that certain structural or functional components are destroyed or disarranged by aging. A similar effect of ageing on particles from rat skeletal muscle has been shown by Nason to be reversed by lipid materials (21). This ageing effect as well as the spectral evidence for other than the usual heme and flavin components in the particles provides new leads to an understanding of their enzymatic properties. The discovery of a quinone in the ETP of beef heart and Azotobacter should lend emphasis to this viewpoint (7).

Summary

The separation of three distinct types of particles from cauliflower bud homogenates is described. They are 1) a grey fibrous material, 2) brown-yellow particles with enzymatic activities and cytochrome components corresponding to those of mitochondria, and 3) light yellow particles occurring in fluffy and packed forms with enzymatic activities and cytochrome components associated with microsomes.

The enzymatic activities investigated were: DPNH oxidation by oxygen, cytochrome c, and indophenol, oxidation of succinate by oxygen, and oxidation of reduced cytochrome c by oxygen. DPNH oxidase is concentrated in the mitochondrial particles and is inhibited by cyanide or antimycin whereas the DPNH cytochrome c reductase is concentrated in the light particles and is not sensitive to antimycin.

The effect of phosphate on the requirement for

cytochrome c for DPNH oxidase is described. DPNH oxidase activity declines on storage and is restored by addition of serum albumin. New DPNH and succinic cytochrome c reductase activity appears in medium particles treated with phosphate and ethanol.

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FACTORS INFLUENCING ABSCISSION[1,2,3]

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The hormonal concept of abscission control was spearheaded by the suggestive experiments of Laibach in 1933 (15) and then more specifically established by the experiments of La Rue (17) in which he found that indoleacetic acid, pollen extracts, or urine could delay the abscission of debladed petioles. Since that time, numerous reports have added substantial support to the idea that auxins control abscission through an inhibitory influence (1, 10, 19, 25). More recently the concept of hormonal control has been extended to include auxin promotion of abscission as well (1, 11).

A useful tool for studying the effects of various substances on abscission has been the petiole explant test described by Addicott et al (4). This test with modification has been used subsequently by a number of workers: by Livingston (18) to study the effects of ethylene, sucrose, and auxins on abscission; by Carns (6) to study the influence of metabolic inhibitors; by Osborne (21) to seek a natural factor which promotes abscission; and by Gaur and Leopold (11) to study the quantitative effects of auxin on abscission.

It has been observed in this laboratory that explant tests performed under similar conditions but at different times showed a considerable degree of variation and in fact in some tests no abscission occurred at all. The following experiments were undertaken to establish a more reliable explant test and to clarify some of the factors influencing abscission. Interactions between leaf age, substrates, light and auxin are described.

MATERIAL AND METHODS

Seedlings of *Phaseolus vulgaris* L. var. Red Kidney, were grown in flats in the greenhouse on a 16-hour-day. Daylength was extended with incandescent lamps giving approximately 70 ft-c at plant level.

Explants 1 cm in length were taken from the primary leaves to include 5 mm of petiole and 5 mm of pulvinus tissue. These explants were placed in 5-cm Petri dishes, basal end down, to a 4-mm depth in 1 % agar with or without addenda. There were 20 explants per treatment. Each experiment reported here was repeated in its entirety at least 3 times and the results are consistent with 35 other experiments encompassing variations on these reported ones. Naphthaleneacetic acid (NAA) was incorporated into the agar as its ammonium salt. No organic solvent was used to dissolve the auxin.

Light treatments given to the explants involved a 12-hr day with 300 ft-c light intensity supplied by fluorescent lamps. The dark treatments were kept in a dark cabinet, but were exposed briefly to incandescent light during the reading of the test. All explants were kept at a controlled temperature of 18° ± 2° C.

TABLE I

METHOD OF SELECTING PLANT MATERIAL

DESIGNATION OF LEAF STAGE	DAYS FROM PLANTING	REMARKS
A (Very young)	14	Primary leaves still expanding; 5 to 7 cm in width.
B (Young)	17–19	Primary leaves fully expanded; 10 to 12 cm in width; 1st trifoliate leaves commencing to expand.
C (Medium aged)	24–28	Primary leaves healthy; 1st trifoliate fully expanded; 2nd trifoliate leaves almost fully expanded.
D (Senescing)	31–41	Primary leaves showing visible signs of senescence—yellowing between the veins.

The achievement of abscission was determined visually. As an explant approaches the final stages of abscission there is less absorption of light in the abscission zone so that the zone looks "whitish" while adjacent tissue is still green or yellow and opaque.

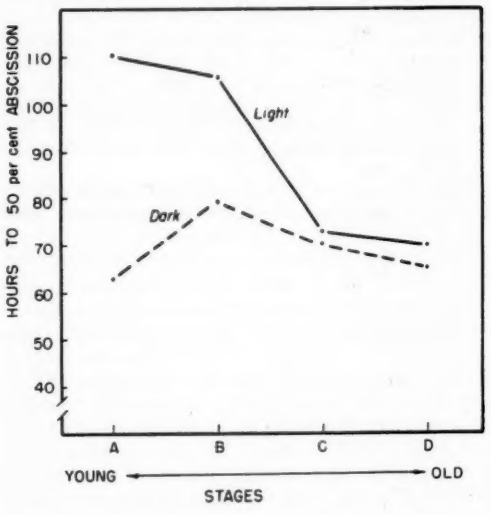

FIG. 1. Natural tendencies for abscission of different age explants in light and dark.

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[2] Journal Paper No. 1120, Agricultural Experiment Station, Lafayette, Indiana.

[3] The work was supported in part by a grant from the Rockefeller Foundation and in part by a grant from the Purdue Research Foundation.

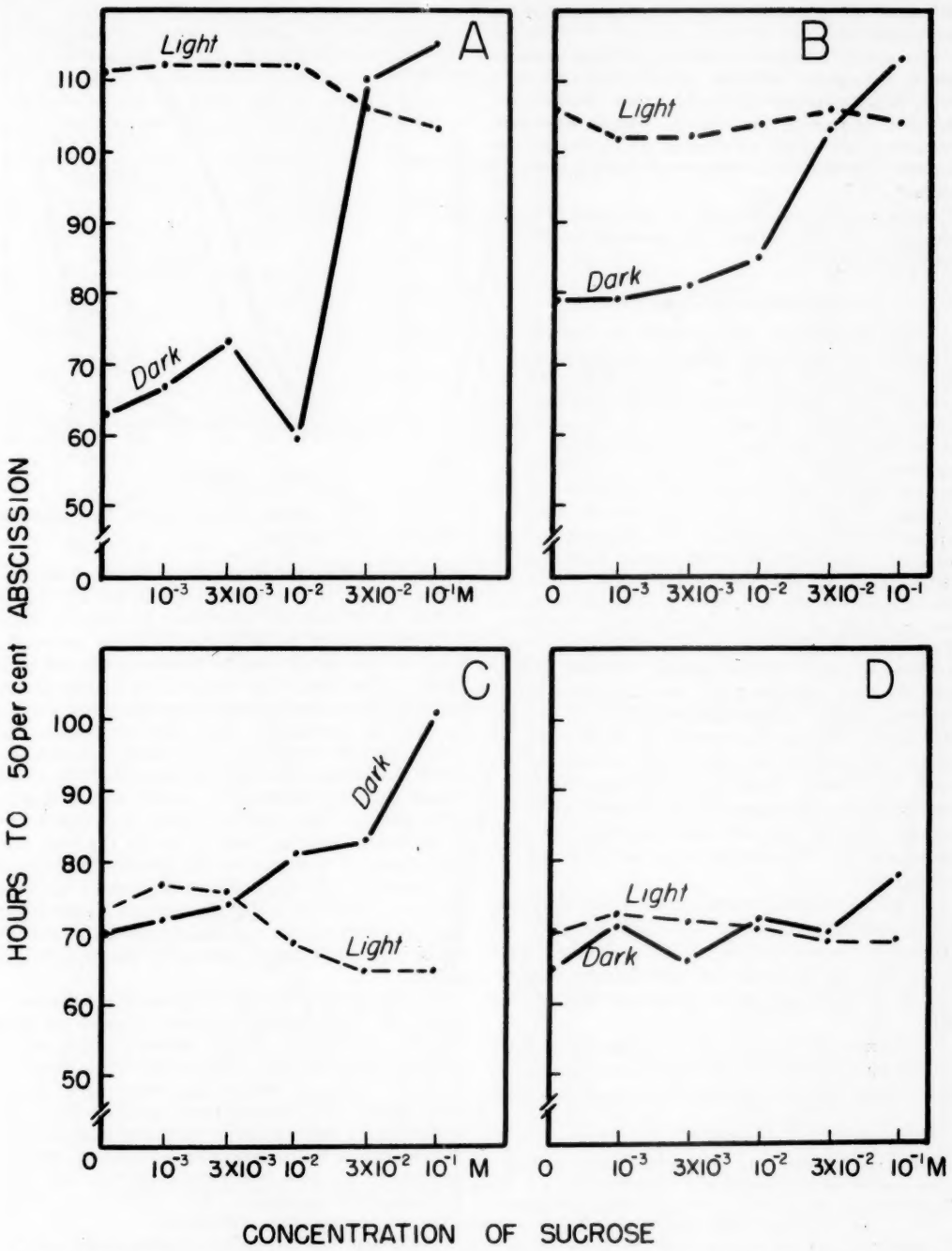

FIG. 2. The effect of sucrose on the rate of abscission of explants of different ages in light and dark: (A) from young expanding leaves; (B) young but fully expanded; (C) medium age; (D) and senescing leaves.

At this stage a slight pressure applied to the pulvinus 90° from the grooved side will readily bring about separation.

To obtain CO_2-free conditions, normal air was forced through a soda-lime column (3.1 × 60 cm), 10 % KOH, distilled H_2O, and finally through the chambers containing the explants. Flow rates were adjusted to give about 1.5 changes of CO_2-free air per minute. The soda-lime column was changed every 24 hours.

Plant material was selected for uniformity of physiological age. The method for selecting material is outlined in table I.

Experimental Results

The Age Factor: To approach the problem of variability of the explant test the influence of physiological age of the tissue was investigated. The results of a leaf age experiment are shown in figure 1. It can be seen that the normal tendency for abscission in the dark does not vary a great deal with age. The greatest variation in the time required for 50 % abscission between age classes is only 16 hours. If the explants are kept in the light there is a striking difference between the abscission rate of young and old material. The difference in this case is as great as 40 hours. These age differences are quite reproducible in the light, but in the dark there is considerable variation especially in medium aged materials. Occasionally explants from this age group will take longer than 90 hours to attain 50 % abscission. Usually, if they take this long, abscission will not take place before autolysis occurs. These explants which will not abscise in the dark can be made to do so by light. A further observation has been that if explants are taken from leaves which have been growing under conditions that prevented the accumulation of carbohydrate reserves, e.g., low light intensity and high temperatures or high nitrogen, they have a slower abscission rate.

From the foregoing experiments it is seen that light has an inhibitory effect on the abscission of young explants which becomes less and less as the tissue ages. Healthy explants from any age material have approximately the same abscission rate when kept in the dark.

Influence of Substrate: From the above observations we surmised that if explants were taken from leaves under certain environmental conditions they might be slow to abscise due to a limited amount of substrate. Tests were conducted with the addition of sucrose to the four leaf age classes as shown in figure 2. From data on explants of very young leaves (fig 2 A), sucrose can be seen to produce a marked inhibition of abscission in the dark at high concentrations. In light, the lack of a sucrose inhibition was evident in all of the age classes. The difference between the dark and light treatments is striking. Sucrose inhibits abscission in the dark most markedly in young tissues and this inhibition is weaker and weaker in older tissues. Light produces about the same degree of inhibition in young material as do the high concentrations of sucrose. As the physiological age of the material increases, as shown in figures 2 B, C and D, light too loses its inhibitory influence. Thus, either light or added sucrose can inhibit abscission, and the inhibitions are roughly similar in the various age classes.

Table II

Effect of CO_2-free Air on the Abscission of the Explants (Hrs to 50 % Abscission)

Addenda	Light (300 ft-c)		Dark	
	Normal air	$-CO_2$	Normal air	$-CO_2$
0	117	71	77	73
3×10^{-2} M sucrose	116	97	109	96

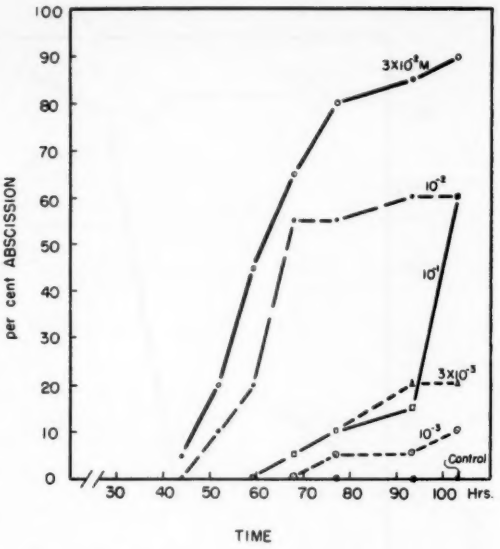

Fig. 3. The influence of sucrose on the abscission of medium age explants.

If the influence of light is primarily a photosynthetic one, then explants exposed to light but in a CO_2-free environment should abscise similarly to explants kept in the dark. This is the case as shown in table II. Explants from young beans placed in the light under CO_2-free conditions attained 50 % abscission in 71 hours which is very similar to the rate of abscission in the dark with or without CO_2, whereas explants in the light with normal air took 117 hours to reach 50 % abscission. The additions of sucrose essentially erased these differences.

As shown thus far, sucrose applied in the dark inhibited abscission, but in occasional tests when natural abscission does not take place or is very slow, sucrose will promote abscission as shown in figure 3. In this experiment the controls had attained only 10

% abscission after 120 hours with the remaining explants showing visible signs of autolysis. Explants which would be unable to abscise were stimulated to do so by sucrose. When the abscission rate of the most promotive sucrose concentration (3×10^{-2} M) is compared to the abscission rate of vigorous explants in the same age group (control in fig 2 C) it can be seen that sucrose stimulates weak explants only up to the rate of abscission exhibited by the more vigorous material. It appears, therefore, that additions of sucrose to explants very low in substrates can permit abscission processes to proceed where they would otherwise not occur. High concentrations can inhibit abscission as shown above.

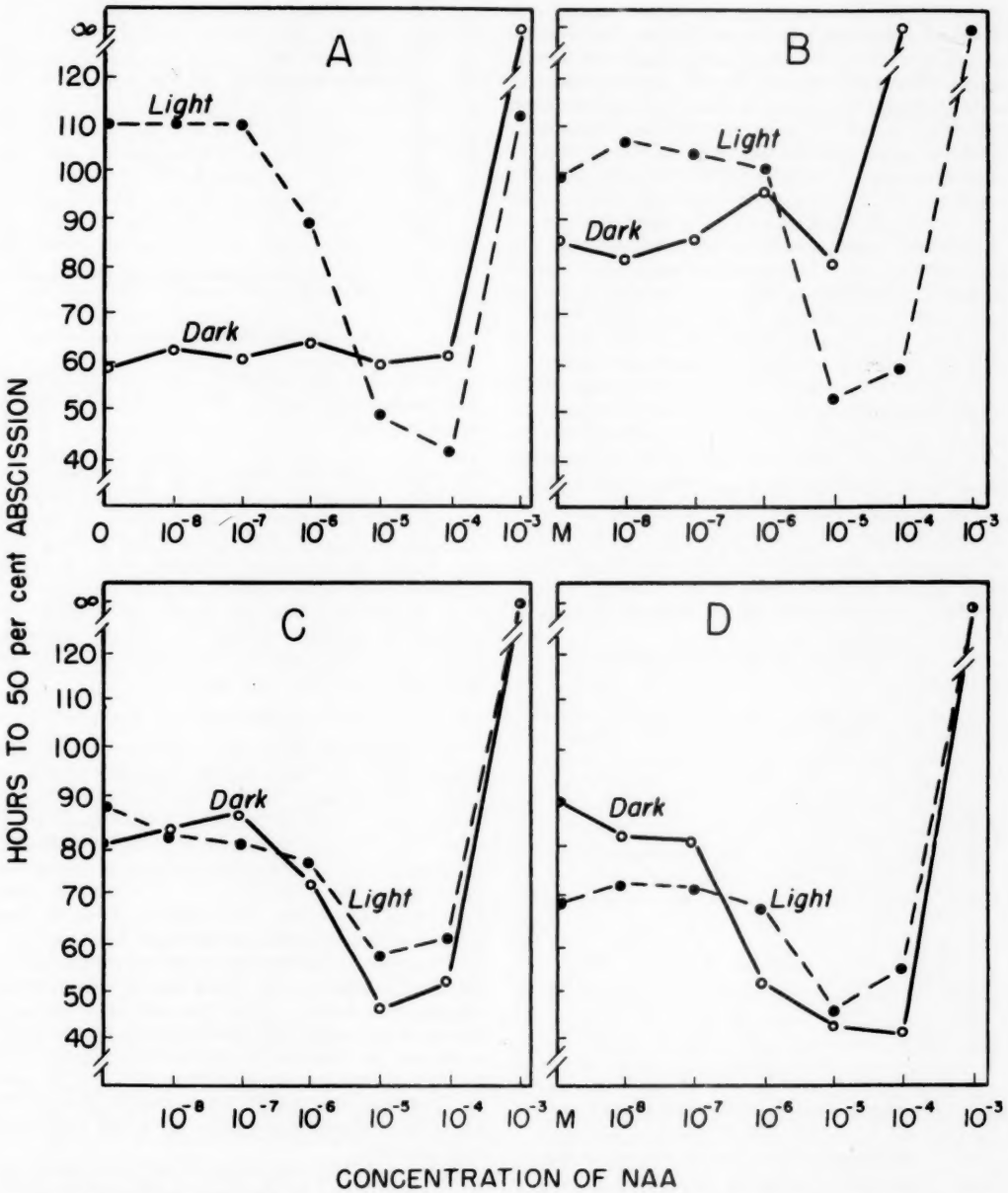

FIG. 4. The effect of auxin (NAA) on the rate of abscission of explants of different ages in light and dark: (A) from young expanding leaves; (B) young but fully expanded; (C) medium age; (D) and senescing leaves.

INFLUENCE OF AUXIN: From test to test we have observed a marked degree of variation in the abscission responses of explants to auxin. It was not clear what part of this could be ascribed to physiological age or to limited substrate. A series of NAA concentrations was applied to explants taken from the different aged materials. Identical experiments were conducted in light and dark. Data obtained from a typical series of experiments are shown in figure 4. There is a remarkable difference between the response of young explants to NAA in the light and in the dark. This difference becomes less and less with age until it finally disappears in the senescing material. No promotion of abscission was obtained in the dark in the younger material (fig 4 A), but large promotions in medium aged material (fig 4 C) and possibly even greater promotions in senescing sections (fig 4 D). With the explants receiving light, strong promotions are obtained with all age classes though decreasing with age. This changing sensitivity to auxin is shown graphically in figure 5. In this figure are presented the differences in time to abscise between the most promotive concentration of NAA (10^{-5} M) and the control as an index of promotive sensitivity to auxin. In the dark, explants from young leaves show no promotion with NAA, and older sections show a rising promotion up to 47 hours. In the light, promotive effects of NAA were greatest in the youngest material and decreased with age, from 61 to 21 hours.

To establish whether light influences auxin responses through a substrate effect, an additional type of experiment was conducted using medium aged material which could not abscise without added substrate. With this material we tested the response to auxin in the dark, in the dark plus 3×10^{-2} M sucrose, and in the light. As shown in figure 6, when explants are placed in the dark plus sucrose, auxin effects are similar to those in light. The explants in the dark without added sucrose never attained 50 % abscission either with or without auxin. We can conclude from these experiments that the nature of the abscission response of explants to NAA is strongly dependent on the age of the tissue and the conditions of the test with respect to sugar and light.

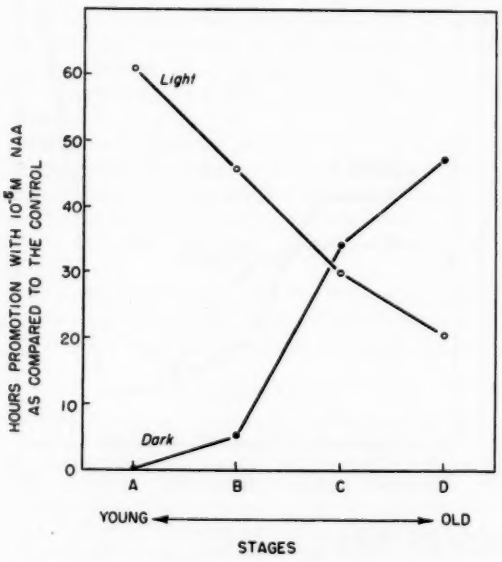

FIG. 5. The sensitivity of different age explants to auxin (NAA) in light and dark.

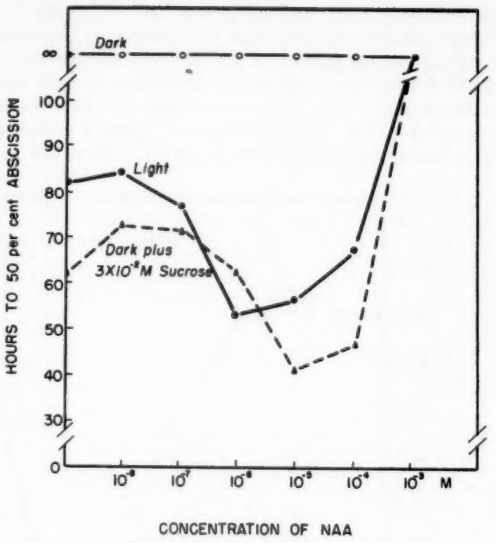

FIG. 6. The effect of auxin (NAA) on medium age material in the dark, in the dark plus 3×10^{-2} M sucrose, and in the light.

DISCUSSION

It is evident from the experiments described herein that sugar has a marked influence on the abscission of explants. Brown and Addicott (5) reported that a droplet of 10 % sucrose solution applied daily to bean explants would inhibit abscission. Livingston (18) immersed citrus explants momentarily in sucrose solutions of 20, 10, 3, 1 and 0.3 % and found that the explants were inhibited by all of them. Similar inhibitions with sucrose have been confirmed in the present experiments (dark treatments in fig 2). In addition to the inhibition, we have shown (fig 3) that explants taken from environmental conditions which cause a low endogenous substrate reserve, can be *stimulated* to abscise with sucrose. The optimum concentration of sucrose (3×10^{-2} M) gave an abscission rate which approximated that of the healthiest explants in the same age group (dark control, fig 2 C).

With respect to sugar substrates, the general picture seems to be that abscission is affected by sucrose in the following manner: if the tissue is deficient in sugars, abscission cannot proceed; moderate amounts

permit the fastest abscission; and only very high levels of sugar inhibit abscission in the manner reported by earlier workers.

It is not surprising that low endogenous concentrations of substrate will limit abscission since it has been affirmed several times that this process is an active process requiring energy (2, 7, 22). Only speculations can be offered to explain the difference between moderate and high levels of substrate.

The role of light in inhibiting the abscission of explants can be attributed principally to photosynthesis. Our basis for this interpretation is that addition of sugar will inhibit abscission to approximately the same degree as will light, especially in young tissue. Also, explants placed in light but with a CO_2-free atmosphere show a rate of abscission which approximated that of explants kept in the dark (table II). Furthermore, the decline in the light inhibition with the age of the tissue is suggestive of the decline in the photosynthetic capacity of leaves as they age (9, 20, 24). On the other hand, there appear to be differences between the light and sugar effects. This is most evident in figure 2 C which describes the response of medium aged petioles to light and sucrose. The fact that sucrose can inhibit when light cannot suggests that light may have other influences on abscission. It is interesting in this connection to note the reports by Lane and Hall (16) and Hall and Liverman (13) in which they found that light increases the effectiveness of chemical defoliants.

With these various responses to sugar substrate in mind, we may be able to shed some light on observations that have been made with intact plants. It has been repeatedly observed that low carbohydrates levels lead to leaf, flower and fruit abscission (2). Chandler (8) has proposed that competition for carbohydrates is responsible for the June drop of apples; he reduced this abscission by ringing which increases carbohydrates above the girdling cut. Lane and Hall (16) found that additions of carbohydrates to chemical defoliants sprayed on cotton in the greenhouse increased the effectiveness of the defoliant. Conversely, Hall (12) found that high levels of endogenous carbohydrates in cotton resulted in a decreased effectiveness of the defoliant. These apparently conflicting observations may be resolved by our experiments.

As has been suggested by Juhren and Went (14), high sugar levels may have a tonic or protective effect on plant cells. Plants which have an adequate sugar reserve may be resistant to chemical defoliation by having a greater capacity for detoxication of the foreign material.

The conditions of the explant test and the age of the tissue used make a great difference in the type of abscission response obtained with additions of auxin. One of the most evident means of explaining the difference between the responses in light and dark is the metabolism of endogenous auxin. The fact must not be overlooked that the auxin supplied is augmented by the amount already in the tissue. Shoji et al (23) have shown that the ether-extractable auxin content of bean leaves decreases with age, hence with age one would expect greater promotions with auxin. This is the case with explants kept in the dark, but in the light greater promotions of abscission are obtained with younger material. This change in sensitivity to NAA between light and dark can be in part ascribed to a substrate effect. From a review of figure 4 it can be seen that light, which seems to influence abscission through sugars, inhibits the abscission process in the younger tissue. The promotive concentrations of auxin shorten the time for 50 % abscission to approximately the same level regardless of the age of the tissue. This may be interpreted to mean that light inhibits the abscission process but when the explant is confronted with a promotive concentration of auxin, the light inhibition is lost. This results in a wide degree of difference in the abscission time in the young tissue between the control and the most promotive concentration of auxin. This degree of difference narrows with increasing age of the tissue and the lessening of the inhibitory effect of light.

In view of the experiments reported herein, some reservations must be kept in mind concerning the earlier studies which made use of the explant test. Results obtained from the influence of auxin on the abscission of explants have supplied the basis of the "auxin gradient theory" as proposed by Addicott et al (2, 3). The theory holds that if the auxin concentration is higher on the proximal side of the abscission zone, abscission is accelerated. By manipulation of the test conditions, the present study shows that proximal applications of auxin can produce both promotions and inhibitions, or only inhibitions of abscission (fig 4). Again, Gaur and Leopold (11) have used explant abscission tests in support of the concept that the two-phase auxin curve of promotion and inhibition best describes the auxin influences. The same caution is needed here, for under the conditions of their explant test (medium aged material kept in the dark) factors other than auxin can limit abscission. Osborne (21) has used a similar test to demonstrate that extracts from senescing leaves contain an abscission promoting factor other than auxin. Before making reliable deductions about her experiments, it will be essential to show that the factor is not simply sugar.

Summary

The conditions for use of petiole explants from red kidney bean for abscission tests have been examined in some detail. The following conclusions have been drawn: 1) The time for abscission of explants is greatly influenced by the age of the leaf, and the presence of light, sugar, and auxin during the test. 2) Sucrose was found to stimulate the abscission of explants low in endogenous substrates under conditions of darkness, and to inhibit abscission at high concentrations in all but older explant samples. 3) Auxin (naphthaleneacetic acid) was found to promote

abscission at low concentrations (e.g., 10^{-5} M) and inhibit at high concentrations (e.g., 10^{-3} M) within a wide variety of conditions and ages. The promotion effect was not obtained with young explants held in darkness, or with older explants which had a low carbohydrate reserve unless substrate was added. 4) It is concluded that auxin generally has a two-phase action on abscission, but that its action is mediated by leaf age, light, and the supply of substrates such as sugar.

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GROWTH RESPONSES OF CRUCIFERS TO INDOLEACETIC ACID AND INDOLEACETONITRILE[1]

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Housley and Bentley (10) have summarized the problem of indoleacetonitrile (IAN) activity in plant growth. Although there is widespread belief that growth responses to IAN are in reality responses to the indoleacetic acid (IAA) derived from it, before final judgment concerning the physiological role, if any, of IAN can be rendered, it would seem that careful study should be made of some species in which IAN occurs naturally. This paper reports some of the initial efforts in such an attempt.

The only reports of extensive comparisons of IAA and IAN are those of Bentley's group in England using Avena coleoptile sections. It is the purpose of this paper not only to compare the effects of IAA and IAN on crucifers but also to contrast these results primarily with those of the English group.

[1] Received June 4, 1957.

Materials and Methods

Hypocotyls from etiolated seedlings of the following crucifers were used: cabbage (*Brassica oleracea* var. *capitata* L. horticultural strain Golden Acre), radish (*Raphanus sativus* L. horticultural strain Crimson Giant), and turnip (*Brassica rapa* L. horticul-

tural strain Purple Top White Globe). For comparative purposes, etiolated coleoptiles of *Avena sativa* L. strain Victory were used.

Seeds were planted in rows in polystyrene trays of sand. Seedlings were grown and experiments were performed in a room that was maintained at a temperature of 24° C and a relative humidity near 85 %. The room was kept dark except for occasional red illumination from incandescent safelights (Rubylites of 4 C.P.). Germination of the crucifers was somewhat uneven; however experiments were started when most of the seedlings averaged about 30 to 35 mm in height —cabbage requiring about 5 days, radish and turnip, 4 days. Only those within 5 mm of the average were used. With a cutter of the author's design, one 10-mm section was cut from each hypocotyl beginning, as closely as could be determined in the dim red light, 5 mm below the cotyledonary node. For Avena, 10-mm sections were cut beginning 5 mm below the undecapitated tips of 25- to 35-mm coleoptiles. As standard procedure 10 sections were floated on 20 ml of test solution in each 20 × 100-mm Petri dish. When testing extracts and on a few other occasions 5 ml of solution in 15 × 60-mm dishes were used, the smaller volume not giving significantly different results.

The author is well aware that both final length after some arbitrary period of time and growth rates during that time are influenced by many factors including the composition of the basal medium. The test solutions used, except to test effects of pH and buffers, contained no additives other than regulator (or/and extract) and the NaOH or HCl necessary to give a pH of 6 for the following reasons: Radish hypocotyl sections responded essentially identically to IAA and to IAN in 0.005 M sodium maleate solutions of initial pH's of 5 and 6 (final pH's of 5.3-5.4 and 6.2, respectively); however optimal 24-hour growth for both regulators was 25 % less in this buffer than with none, initial pH adjusted to 6 (final pH 6.6 to 6.9). Bentley's group did not adjust initial pH; however, as will be indicated later, this proved important only with the highest IAA concentrations used. Sucrose was not added primarily because the English group, with whose work this was to be compared, did not do so.

Lengths of sections were determined either by direct measurement or photographically as previously described (14). The growth in water alone was subtracted from total growth before plotting the graphs; however the values for water growth appear in the legends. It may be noted that growth in water of crucifer hypocotyls is much less than that of Avena coleoptiles. Greater variability often was found among crucifer sections of a test dish than usually reported or found by the author for Avena; nevertheless average values were quite consistent and reproducible. Representative results have been chosen for presentation in this paper.

Cabbage extract preparations tested were prepared using the following procedure: Freshly harvested etiolated hypocotyls were macerated with mortar and pestle, 1 *N* HCl being added to give pH 2. Following high-speed centrifugation the supernatant, which contained the significant bulk of growth stimulating activity, was decanted and brought to pH 9 to 12 by adding 1 *N* NaOH. This alkaline aqueous supernatant was extracted 4 to 5 times with redistilled, peroxide-free ethyl ether to produce the neutral fraction. The aqueous portion was reacidified with HCl to pH 2 to 3 and extracted 4 to 5 times with ether to produce the acid fraction. The water was frozen out of both neutral and acid ether fractions in a deep freeze. The ether extracts were decanted into distilled water in beakers in a water bath of about 55° C and heated for one hour after visual disappearance of the ether. The now aqueous fractions were adjusted to pH 6 as necessary and diluted and tested as desired.

All water used in preparing test solutions was redistilled from Pyrex after distillation from a tin-lined still and passage through an Amberlite MB-2 column. The IAN was synthesized using a slightly modified procedure of Henbest, Jones, and Smith (9). The final steps in purification of the IAN were completed in the Department of Chemistry at the State University of Iowa under the direction of Prof. S. Wawzonek.

Results and Discussion

To establish common ground, present another base for comparison of results obtained with crucifers, and point out a pH effect mentioned earlier, it is desirable to compare the results of Bentley and Housley (2, 3)

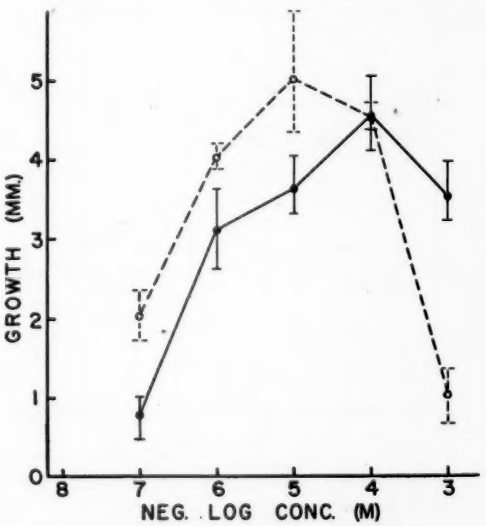


Fig. 1. Twenty-four-hr growth of Avena coleoptile sections in IAA (solid lines) and IAN (broken lines). Each point is the mean of 4 dishes from 2 trials. Vertical lines show extremes of single dish means. Water growth (subtracted before plotting) was 3.6 mm.

with those obtained for Avena by the author. Figure 1 shows the latter, which, with one major exception, closely resemble the published results of Bentley and Housley. The exception is their finding that after 24 hours the average length of sections in 5.7×10^{-4} M IAA was about the same as that for water controls. Initially growth was greater in IAA but this was followed by shrinkage. These workers concede that this might be related to the low pH of 4.1 measured for this concentration however they point out that the connection is not clear since no shrinkage of sections occurred in succinic acid solution at pH 3.5. That this is related to low pH is indicated in figure 1 by the lack of or at least low toxicity at high IAA concentrations when the initial pH is adjusted to 6. Low pH itself may not be toxic, for Nitsch and Nitsch (15) reported greater growth of Avena coleoptile sections in sucrose and buffer with and without IAA (2.9×10^{-7} M) at pH 4 than at higher pH values. The data of Bonner and Foster (4) show little toxicity to Avena coleoptiles even of high concentrations of IAA at the relatively low pH of 4.5 in the presence of sucrose and buffer. It is possible that sucrose and/or buffer have some protective value against low pH. Still, it seems certain that the toxicity of high IAA concentrations reported by Bentley and Housley (2, 3) are either related to low pH or anomalous.

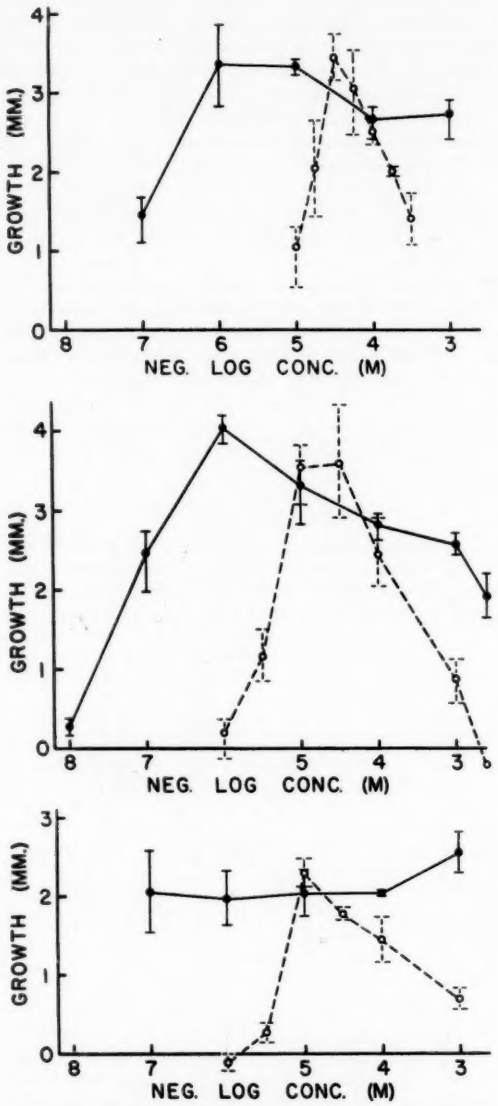


FIG. 2. Twenty-four-hr growth of various crucifer hypocotyl sections in IAA (solid lines) and IAN (broken lines). Each point is the mean of 1 to 5 dishes from 1 to 3 different trials. Vertical lines show extremes of single dish means. *Upper,* cabbage with water growth of 0.6 mm. *Middle,* radish with water growth of 0.9 mm. *Lower,* turnip with water growth of 0.4 mm. Water growth subtracted before plotting.

A low pH not only increases the proportion of undissociated IAA, in which form IAA is presumably more readily absorbed, but also seems to have other effects. Reinhold (17) has found that pH effects do not parallel the dissociation of IAA and that pH and regulator absorption are not clearly related. Van Overbeek (21) has recently and briefly discussed this problem. In this connection it might be mentioned that radish hypocotyl sections grew about half as much in 0.006 M citrate-phosphate (1 : 2) buffer at pH 4.5 as at 7.0, although, as mentioned earlier, they grew about the same in maleate buffer at pH's 5 and 6. About all that can be said at this point is that the effects of pH and buffers are not limited to regulator penetration and that the dependence of regulator absorption on pH is not clear.

With the growth curve for IAA, as shown in figure 1, essentially like that for IAN over the entire concentration range except for displacement to the right, any necessity for invoking different inhibitory mechanisms at high concentrations as was done by Bentley and Housley (2, 3) is obviated. The possibility that high concentrations of both IAA and IAN may possess general or non-specific cellular toxicity unrelated in a direct way to growth mechanisms in addition to probable self-competitive inhibition should not be disregarded.

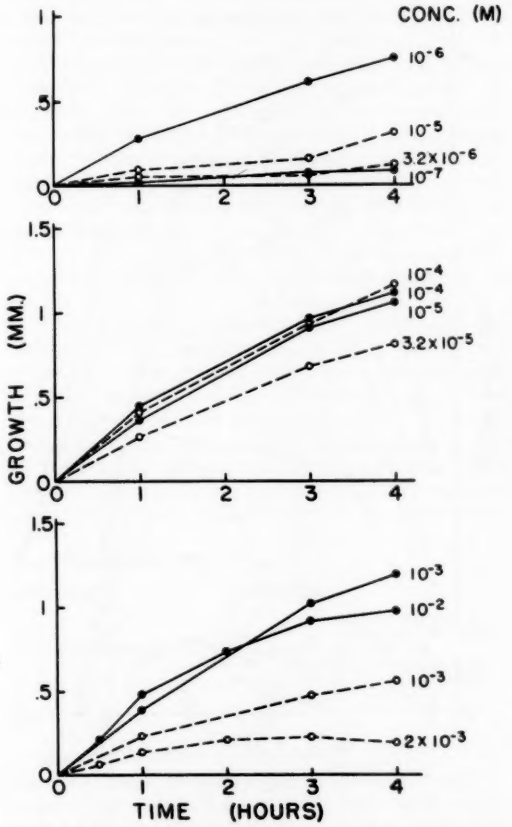
The patterns of growth responses among the crucifers tested, as shown in figure 2, while quite different from those of Avena, are similar to each other except for the response of turnip to IAA. The nearly equal growth of turnip hypocotyl sections over a 10,000-fold IAA concentration range is difficult to understand, though a partial explanation may be indicated by results to be presented later. Germination of the turnip seeds was not uniform, the diameter of the hypocotyls was small, and the sections tended to curve markedly in the test solutions; therefore work with turnip was not continued.

There are several differences between the growth curves for the crucifers and for Avena. Crucifers respond to lower concentrations of IAA. The optimal concentration of IAA for crucifers is 10^{-6} M; for Avena, 10^{-4} M. The growth curves for crucifers do not decline as rapidly at supraoptimal concentrations of IAA as does that for Avena. Crucifers are less sensitive to low concentrations of IAN than Avena; however, the concentrations producing maximum growth are the same. The growth curves for crucifers span less of a concentration range of IAN than does that for Avena.

With the above noted differences in comparative 24-hour growth responses of Avena and crucifers to IAA and IAN, it seemed of interest to compare the growth rates of one of the crucifers with those of Avena reported by Bentley and coworkers (3, 12). These investigators found no appreciable differences between growth rates induced by IAA or IAN at any but the highest concentrations used, if comparisons are made between concentrations giving nearly equal final lengths.

If IAN possesses activity only after hydrolysis, the growth rates observed throughout the entire period of growth would be expected to vary from those of IAA only as a result of side effects, such as general toxicity, or sufficiently slow conversion to cause a lag period. However, if IAN possessed activity without hydrolysis, it would seem logical also to expect nearly the same growth curves as for IAA because of the structural similarity of the two molecules. Bonner and Foster (4) found both IAA and 2,4-D to give linear growth rates that are apparently strictly comparable even though no assumption of conversion of the 2,4-D is made. Without the operation of substantially different growth mechanisms or of side effects or lag periods, one would expect active compounds to produce quite comparable growth rates over long periods of time.

The growth rates of radish hypocotyl sections for a range of concentrations of IAA and IAN during 24 hours are shown in figures 3, 4, and 5. As was mentioned earlier, the values plotted represent the differences between growth in water and in regulators.



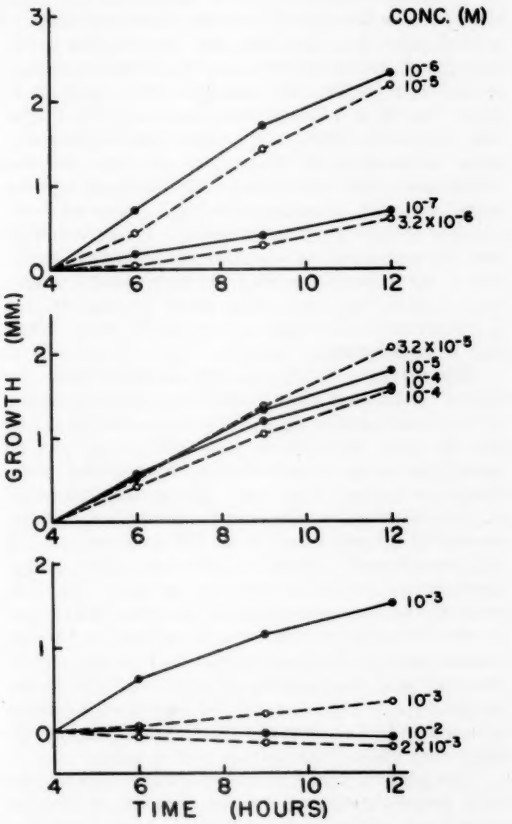


FIG. 3 (*left*). Growth rates of radish hypocotyl sections in IAA (solid lines) and IAN (broken lines). Water growth (subtracted before plotting) given in figure 6. 0 to 4 hrs.

FIG. 4 (*right*). As figure 3 except 4 to 12 hrs.

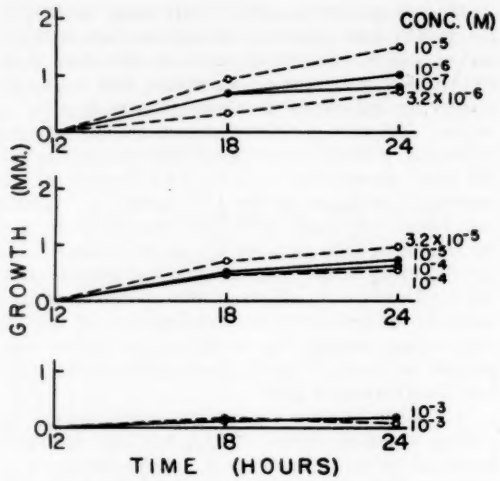


FIG. 5. As figure 3 except 12 to 24 hrs.

In this connection it should be noted that the negative slopes in these graphs do not reflect shrinkage of sections—shrinkage never having been observed—but only growth rates less than those of control sections in water. To enable more accurate representation of early growth and better comparison of actual rates and changes in rates, the curves were broken into three figures covering the periods of 0-4, 4-12, and 12-24 hours; and the figures were broken into three parts, grouping concentrations that produced comparable 24-hour growth. Although it was necessary that the scales for the abscissas in the figures be different, the scales of the ordinates were ajusted so that equal growth rates have equal slopes throughout, e.g., a growth rate of 0.5 mm per hour would have a slope of 1 in each figure.

The results are about the same as those found for Avena (3, 12) and what would be expected according to the preceding discussion. The apparent lag period for the first three hours with 10^{-5} M IAN is an anomalous result as indicated by other similar trials. Using fewer dishes and larger photographs taken 15, 30, and 60 minutes following transferral of sections, the initial growth rates in 10^{-6} M IAA and 10^{-5} M IAN were found to be nearly identical. Both showed slight lag periods of 30 minutes or less. With the bulk of the data showing that in both radish and Avena the initial growth rates in response to IAN do not lag measurably behind those to IAA, no conclusion regarding the necessity of conversion of IAN can be reached. It can be concluded that if such conversion is required, it occurs with sufficient rapidity to escape detection by measuring growth rates.

The possibility of high concentrations of IAA and IAN possessing general cellular toxicity in addition to probable self-competitive inhibition has been suggested. Evidence for such toxicity is illustrated in figures 3 and 4 in the rapid reduction from a high to an extremely low growth rate at the highest concentrations of both IAA and IAN. Toxicity symptoms appear at a lower concentration of IAN than of IAA. Initial growth rates in IAA continue to rise with increasing concentration, but this is not true for IAN. One more evidence of general cellular toxicity of IAN was the flaccid and water-soaked appearance of the ends of radish sections in 2×10^{-3} M IAN, the only concentration in which this was noted. Housley et al (12) found high concentrations of IAA and IAN also to exhibit toxicity symptoms in the growth rates of Avena coleoptile sections, although, as has been pointed out, their reported IAA toxicity seems related to low pH.

The data of figures 3, 4, and 5 are combined and plotted as a function of regulator concentration in figure 6. This emphasizes the limitations of results of the kind presented in figures 1 and 2 since the concentrations producing maximum growth and the shapes of the curves change with time. The points of maximum growth for IAA at the different times plotted on figure 6 sweep from lower right (1 hr, 10^{-2} M) to upper left (24 hr, 10^{-6} M) and for IAN from

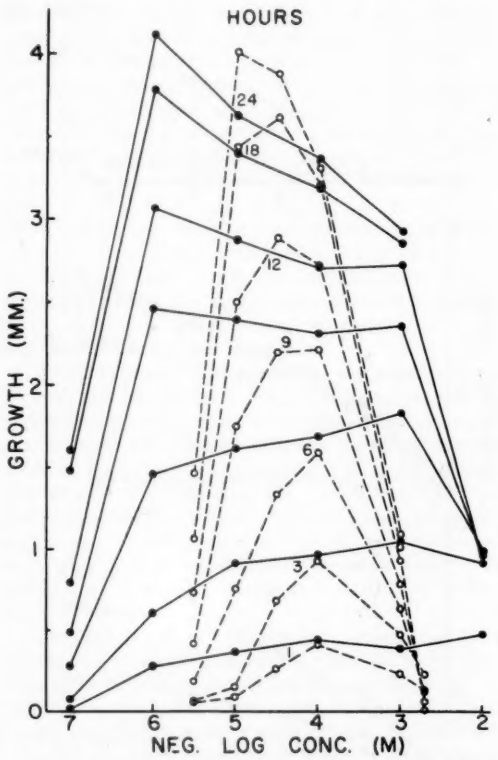


FIG. 6. Growth of radish hypocotyl sections in IAA (solid lines) and IAN (broken lines) after various periods of incubation. Combination of data from figures 3, 4, 5. Water growth (subtracted before plotting) in mm: 1 hr-0.17, 3 hr-0.22, 6 hr-0.35, 9 hr-0.43, 12 hr-0.46, 24 hr-0.57.

lower center (1 hr, 10^{-4} M) to upper center (24 hr, 10^{-5} M). Both shift to lower regulator concentrations with increasing time; however there is a 10,000-fold concentration change for IAA to only 10-fold for IAN. No appreciable change with time in concentration of IAA or IAN stimulating maximum growth in Avena has been reported (3, 4).

Perhaps most surprising is the plateau of uniform response to such a wide range of IAA concentrations —1000-fold even after nearly 12 hours. These results may help explain the apparently anomalous equal growth of turnip sections for such a wide IAA concentration range referred to earlier and recorded in figure 2. That there are no comparable plateaus in the IAN growth curves reflects a combination of two phenomena: the lack of response of radish sections to low concentrations of IAN and the greater general toxicity of IAN than of IAA at high concentrations. The latter has been discussed, but the former needs to be examined in more detail and in relation to the results obtained for Avena.

An attempt to establish a framework that might serve as a guide for interpretation of the data of this paper and also be in agreement with other available data led to the scheme presented in figure 7.

The potentially active IAA was suggested by and is meant to correspond with the "physical uptake" of Reinhold (17), the "diffusible" and "exchangeable" fractions of Johnson and Bonner (13), and the "outer space" of anionic diffusion of Epstein (6). The concentration of potentially active IAA within tissue is presumably the same as or directly proportional to the external concentration. In contrast to the above, Andreae and Van Ysselstein (1) found the uptake of IAA from external solution to be wholly dependent on aerobic respiration in pea stems.

The metabolic removal is based mostly on the findings of the above authors (1, 6, 7, 13, 17) and others. It is also presumed that the actual utilization of IAA in growth is metabolic, with the IAA so used never returned as again potentially active. The metabolic uptake might by-pass the diffusible internal pool of potentially active IAA in the epidermal cells. However, as pointed out by Epstein (6) in regard to uptake of ions, this could hardly be expected in the internal cells. With an internal pool filled to at least a steady state by diffusion within 20 to 30 minutes (6, 13), inclusion in the diagram of metabolic uptake directly from the external medium seemed superfluous or even misleading.

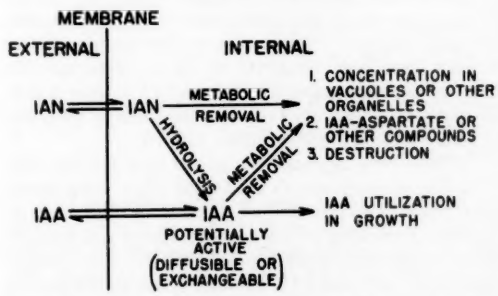


FIG. 7. Tentative scheme of possible dispositions of IAA and IAN in plant sections placed in solutions of these compounds.

Direct utilization of IAN in growth is not included since there is no clear evidence that it occurs; however, reference will be made from time to time to this possibility.

The rates of reactions and even some of the reactions presented in figure 7 certainly vary from species to species. It is this variability that must be used in any attempt to interpret or possibly explain the differences in responses noted. As one example may be cited the lack of significant responses of corn and peas to IAN, which indicates not only absence of direct IAN utilization but also absence of an IAN hydrolyzing enzyme or enzyme system in these species (19). Another example is the variability, both qualitative and quantitative, of metabolic products of IAA found in 12 plant species (7).

The belief, which seems to have become so widespread, that IAN activity in Avena depends on hydrolysis rests not only on proof that hydrolysis does occur but also on the assumption that the internal potentially active concentration of IAA is 3 to 10 times greater in any IAN solution than in an IAA solution of the same concentration. An apparently reasonable explanation has been offered to account for the latter (18)—a greater rate of uptake of IAN than of IAA coupled with considerable IAN hydrolysis.

Throughout the lower concentration range Avena requires about 5 times more IAA than radish for comparable growth. This greater sensitivity of radish could reflect differences in one or more of the actions which add IAA to or remove it from the potentially active pool. Whether movement across the membrane is sufficiently rapid to allow establishment of near equilibrium and not be limiting or is no more than a limiting steady state flow is undetermined. Greater penetrability of IAA through radish than through Avena membranes could be a reason for occurrence of the above phenomenon. Lower non-growth metabolic removal of IAA in radish than in Avena also could account for the greater sensitivity of radish since the effect would be to increase the relative amount of potentially active IAA. The most probable explanation, however, might lie in the relative reactivities in growth utilization of IAA or any complexes formed.

In view of the greater sensitivity of radish than of Avena to low IAA concentrations, it was somewhat surprising to find that radish requires about 100 times as much IAN as Avena for response. From the discussion above it certainly would appear that radish differs considerably from Avena in its ability to produce or maintain from external IAN a pool of potentially active IAA, if in fact it does so at all. If radish membranes are no less permeable than those of Avena to IAA, could they be expected to be less so to IAN? More reasonable would be a greater metabolic re-

moval of the internal IAN or a less concentrated hydrolyzing enzyme or one with a lower turn-over number. Steady state conditions for any hydrolysis that does occur must be rapidly established because, as indicated earlier, no greater lag period in growth response is found than for IAA alone. Still another possibility exists if one is ready to assume that radish membranes are more permeable to IAA than those of Avena. Perhaps a greater proportion of any IAA produced from IAN leaks to the external solution (where the concentration of IAA remains essentially zero) from radish than from Avena. Much of the IAA produced from IAN by Avena has been found in the external solution (18).

The difficulty of evaluating extractable auxins is well known (8). One possible lead in consideration of the hydrolysis to IAA of exogenously supplied IAN would be knowledge of the endogenous concentrations of IAA and IAN. Whether or not IAN is a natural precursor of IAA has not been shown; however the data of this paper show that if IAN is not active per se, it can be a precursor of IAA in hypocotyl cells. The early work of van Overbeek (20) with radish seedlings and the extremely limited growth of sections in water reported in this paper make it appear that any IAN found in hypocotyl cells originated in the upper portions of the seedlings as IAN or one of its precursors. Thus, IAN supplied exogenously in solution might be expected to find the same fate in hypocotyl cells as that originating within the seedlings. This should be true at low external concentrations at least, though Andreae and Van Ysselstein (1) found the patterns of internal metabolism and disposition of IAA in pea stems to be different at low and moderately high external concentrations.

Activity in the Avena coleoptile section test, described earlier, was found in the acid fraction from an aqueous extract of cabbage hypocotyl equivalent to approximately 2×10^{-7} M IAA in the original hypocotyls. The use of extract dilution and admixture with known concentrations of IAA indicated the presence of one or more inhibitors in the acid fraction that strongly inhibited growth of cabbage hypocotyls. They, however, had little effect on Avena coleoptile growth. No attempt has been made to determine the nature of these inhibitors.

The color reaction of this acid fraction in a Salkowski test produced an off shade of reddish pink that had considerable yellow in it as indicated by the absorption spectrum, which was somewhat reminiscent of the curve observed by Platt and Thimann (16) with a mixture of catechol and IAA. The intensity of the color reaction was several times greater than would have been obtained from a pure IAA solution of the concentration indicated by biological activity. Holley et al (10), while reporting IAA in ether extracts of cabbage leaves, also found much color developing that was not attributable to IAA. Although it was not proved that IAA was present in the extract from cabbage hypocotyls, the biological assay and color reaction point to the presence of some active, indole compound; and the fractionation procedure used makes it highly unlikely that it was other than an acid. Housley and Bentley (11) did not find IAA in cabbage leaves; however they stated that any present might have been destroyed during extraction.

The activity in the Avena coleoptile section test of the neutral fraction from the aqueous extract was equivalent to approximately 10^{-5} M IAN in the original hypocotyls, which is about the same as found in cabbage leaves by Henbest et al (9). The results of Housley and Bentley (11) indicate that almost all the activity found in the neutral fraction of an ether soluble extract is attributable to IAN.

The neutral fraction was negative in the Salkowski test and exhibited little evidence for the presence of inhibitors of Avena coleoptile growth, though it inhibited growth of cabbage hypocotyls nearly 100 %.

Since the growth responses of cabbage hypocotyls to 2×10^{-7} M IAA and to 10^{-5} M IAN are more than double the growth in water (fig 2), it seems certain that not all of the extractable auxin is immediately or potentially available for growth. Johnson and Bonner (13) report similar findings with 2,4-D and Avena coleoptiles. This could be interpreted as demonstrating that a continuing supply of regulator from a source external to the growing cell is essential for appreciable continued growth by elongation and that much of the regulator passing into a cell becomes unavailable for growth by elongation, though it conceivably could produce other of the recognized growth regulator effects. This might be expected if the regulator effect in growth by elongation is limited to the loosening of the cell wall, as most recently supported by Cleland and Bonner (5). It is also consistent with the scheme presented in figure 7.

Whether or not the 50 to 1 ratio of IAN to what is probably IAA reflects a limited hydrolysis of IAN is, therefore, uncertain. Such a ratio does, however, correlate quite well with the lesser sensitivity of crucifers to IAN than to IAA and the assumption that IAN must be hydrolyzed to be active in growth.

Analytical studies of the fates of externally supplied IAA and IAN are underway at the present time.

Summary

The 24-hour growth of cabbage, radish, and turnip hypocotyl sections was compared with that of Avena coleoptile sections. The crucifers were more sensitive to IAA but less sensitive to IAN. Whereas the growth response ranges for IAA and IAN were nearly identical for Avena, for crucifers the range of response to IAA was much greater than to IAN.

As had been reported for Avena, the growth rates of radish in response to IAA and IAN were essentially alike. Growth rates changed with time, increasing then decreasing at low concentrations and decreasing at medium to high concentrations. The concentrations of IAA and IAN producing maximum growth shifted to lower values with increasing time up to 24 hours, the shift for IAA being much greater than for IAN.

The concentrations of endogenous IAN and an active, acid, indole compound—presumably IAA—were found to be of the magnitude of 10^{-5} M and 2×10^{-7} M equivalents, respectively.

A scheme indicating the probable or possible fates of IAA and IAN following external presentation of these to plant sections was presented and the data discussed in relation to this scheme. Since several possibilities for explanation of the results exist, whether or not IAN is active in promoting growth before or only after hydrolysis in crucifers cannot now be determined.

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THE RELATION OF OPTICAL FORM TO THE UTILIZATION OF AMINO ACIDS. II. UTILIZATION OF STEREOISOMERIC VARIETIES OF ASPARTIC ACID AND ASPARAGINE BY CARROT ROOT DISKS[1]

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The numerous studies, that have been made, of the use of the amino acids as nitrogen sources for growing plants, embryos, plant tissues, and the limited number of investigations of the utilization of stereoisomeric varieties of amino acids by higher plants were referred to in the first paper of this series by El-Shishiny and Nosseir (3). The recent work of Webster and Varner (11) showed that lupine seedlings absorb and utilize aspartic acid.

The study of the metabolism of the stereoisomers of amino acids in living organisms has increased our knowledge of the range and nature of oxidative and synthetic reactions available to the body. More-

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over, biological differences between these two groups of stereoisomerides illustrate the marked specificity which specializes reactions of living matter. The aim of the present study is to investigate the ability of carrot root disks to absorb and utilize the stereoisomeric varieties of aspartic acid and asparagine.

Materials, Methods and Experiments

The disks were prepared from carrot root *Daucus carota* var. Chantenay. Twenty grams of one-mm thick disks, taken at random from a stock prepared for each experiment, were used for each sample. The samples after being washed for 4 days were transferred into 350 ml of culture solutions. The general procedure and the methods of determination of the various nitrogenous fractions were as already described by El-Shishiny (2) and El-Shishiny and Nosseir (3). The utilized amino acid- and ammonium-N were calculated by substracting the final content of the nitrogen fraction in the disks from the sum of the initial amount of that fraction and that absorbed from the external medium.

The required carrot disk samples for each experiment, after being washed for 4 days in distilled water, were transferred to the culture solutions. The cultures were kept at $25 \pm 0.1°$ C during the experimental period of 24 hours. Experiment I was designed to investigate the uptake and utilization of L- and D-aspartic acid. Hence, duplicate samples were cultured in the following solutions: Distilled water; ammonium sulfate; L-aspartate; L-aspartate + ammonium sulfate; D-aspartate; D-aspartate + ammonium sulfate. The concentration of aspartates and ammonium sulfate in the solutions was 0.0025 M. Experiment II was designed to investigate the effect of D-aspartate on the uptake and utilization of the other enantiomorph when supplied simultaneously to carrot root disks. For this purpose duplicate samples were cultured in the following solutions: 0.0025 M ammonium sulfate; 0.0025 M ammonium sulfate + 0.0025 M L-aspartate; 0.0025 M ammonium sulfate + 0.005 M L-aspartate; 0.0025 M ammonium sulfate + 0.0025 M L-aspartate + 0.0025 M D-aspartate; 0.0025 M ammonium sulfate + 0.0025 M L-aspartate + 0.005 M D-aspartate; 0.0025 M ammonium sulfate + 0.005 M DL-aspartate. For experiment III, which was designed to investigate the uptake and utilization of stereoisomeric forms of asparagine, duplicate samples were cultured in the following solutions: Distilled water; ammonium L-aspartate; L-asparagine; ammonium D-aspartate; D-asparagine; ammonium DL-aspartate; DL-asparagine. The concentration of the solutes was 0.0025 M.

The chemicals used were of the analytical grade unless otherwise indicated. The aspartic acids (P) and asparagines were purchased from L. Light and Co. Ltd. The other chemicals were produced by Merck & Co. Inc. The aspartic acids were neutralized to pH 7 with KOH in experiments I and II, and with NH_4OH in experiment III.

Results and Discussion

Uptake and Utilization of L- and D-Aspartic Acids and Asparagines: Judging from the results depicted in table I, carrot root disks were able to absorb both stereoisomeric forms of aspartic acid from their respective solutions. The absolute amount of amino-N absorbed from L-aspartate was, in all experi-

Table I

Effect of Culturing Carrot Root Disks for 24 Hours in Solutions of 0.0025 M L- or D-Aspartic Acid Alone or in Combination with 0.0025 M $(NH_4)_2SO_4$

Nitrogen fractions	Mg N/100 gm fresh wt of tissue *					
	Control in H_2O **	Changes in the N fractions as compared with controls				
		H_2O		$(NH_4)_2SO_4$		
		L-Aspartate	D-Aspartate	Control **	L-Aspartate	D-Aspartate
Ammonium-N	0.82	+ 0.51 †	− 0.66 †	+ 8.94 †	+ 2.98 ††	+ 1.32 ††
Glutamine-N	1.31	+ 8.32	+ 3.32	+21.10	+22.30	− 3.21
Asparagine-N	4.60	+ 7.03	+ 3.00	+ 4.19	+ 3.31	+ 0.67
Amino acid-N	10.37	+17.65	+16.70	− 2.02	+ 5.77	+14.88
Rest-N	35.70	−14.27	− 7.08	+ 4.58	+ 8.15	− 3.50
Insoluble-N	50.70	+19.90	+ 1.57	− 0.06	+ 3.84	− 1.22
		Absorbed nitrogen				
Ammonium-N	....	....	....	41.5	55.4	30.10
Amino acid-N	....	36.2	16.94	....	30.4	16.20
		Utilized nitrogen				
Ammonium-N	....	− 0.69	0.48	32.40	43.3	19.66
Amino acid-N	....	18.01	− 0.28	1.50	26.1	2.82

* Means of duplicate samples.
** Control tissues are cultured in distilled water and in 0.0025 M $(NH_4)_2SO_4$.
† Compared with control in water.
†† Compared with control in $(NH_4)_2SO_4$.

TABLE II

EFFECT OF CULTURING CARROT ROOT DISKS FOR 24 HOURS IN SOLUTIONS OF 0.0025 M AMMONIUM SULFATE ALONE OR IN COMBINATION WITH L-, L- AND D-, OR DL-ASPARTIC ACID

NITROGEN FRACTIONS	MG N/100 GM FRESH WT OF TISSUE *					
	CONTROL IN $(NH_4)_2SO_4$	CHANGES IN THE N FRACTIONS AS COMPARED WITH THE CONTROL				
		0.0025 M L-ASPARTATE	0.005 M L-ASPARTATE	0.0025 M L- AND 0.0025 M D-ASPARTATE	0.0025 M L- AND 0.005 M D-ASPARTATE	0.005 M DL-ASPARTATE
Ammonium-N	12.01	+ 6.49	+ 1.92	+ 3.56	+ 1.20	+ 5.25
Glutamine-N	16.65	+18.20	+35.30	+18.57	+15.67	+17.56
Asparagine-N	7.08	+ 4.24	+11.48	+ 3.81	+ 6.29	+ 2.87
Amino acid-N	8.43	+ 2.28	+ 3.29	+ 1.91	+ 2.05	+ 2.12
Rest-N	12.60	+ 9.07	+ 4.51	- 0.92	+ 4.87	+ 5.27
Protein-N	74.70	+ 0.44	+ 9.01	+10.73	+ 0.71	+ 6.69
Absorbed nitrogen						
Ammonium-N	48.2	57.7	66.6	51.4	45.2	50.6
Amino acid-N	....	33.7	43.5	33.4	35.0	36.5
Utilized nitrogen						
Ammonium-N	36.60	39.7	53.2	36.3	32.4	33.8
Amino acid-N	- 2.26	29.1	38.0	29.2	30.6	32.1

* Means of duplicate samples.

ments, more than double that absorbed from D-aspartate under similar conditions. A possible explanation for the different rates of uptake of L- and D-aspartate by carrot root disks might be similar to the explanation given for that of the stereoisomerides of glutamic acid by El-Shishiny and Nosseir (3). The uptake of aspartate was accompanied by an increase in the respiration rate of carrot root disks. This result might indicate the dependence of uptake on respiratory energy as suggested by Webster (10) for the uptake of amino acids. Table II indicates that the total amino-N uptake was greatly increased when the concentration of L-aspartate, supplied together with ammonium sulfate, was doubled. On the contrary, there was no material change in the amount of amino-N removed by carrot root disks when the aspartate concentration, in the L-aspartate–ammonium sulfate culture solution, was increased either by adding D-aspartate or by using DL-aspartate.

Table I shows that carrot root disks have marked ability to utilize L-aspartic acid, since as much as 50 % of the absorbed L-aspartate was utilized. The utilization of L-aspartate by carrot root disks was increased in the presence of ammonium-N in the culture solution and also when the concentration of L-aspartate was doubled. The utilization of aspartate by other plant material was also reported by Spoerl (8), Riker and Gutsche (6), Saïd and Younis (7), and Webster and Varner (11). Virtanen and Linkola (9) reported that both L- and D-isomers of aspartic acid were used by pea and clover. The results obtained from the present investigation suggested, however, that D-aspartate, absorbed by carrot root disks, might have behaved as inert metabolite, since the increase of amino acid-N level in the tissue was equal to the absolute amount absorbed (table I). The accumulation of the absorbed D-aspartate might indicate that the prevailing enzyme system is specific to the L-form.

Table III shows that equal amounts of nitrogen were removed from L- and DL-asparagine solutions, but slightly lower amounts were removed from the solution of D-asparagine. It was interesting to find that equal amounts of amino- and amide-N were removed from solutions of asparagine. This might indicate that the asparagine molecules were absorbed intact. This conclusion was substantiated by the fact that neither was ammonia detected in the external media nor was there any significant change in the levels of ammonia in tissues cultured in asparagine solutions. Moreover, almost all of the asparagine-N removed from the external solution accumulated as such in the cells. Another interesting observation was that the total-N removed from the culture solutions of asparagine was greater than from those of ammonium aspartate (table III). The greatest difference was found between the nitrogen absorbed from asparagine and ammonium D-aspartate solutions. This might be due to the slow rate of uptake of the D-aspartate ions and their depressing effect on the absorption of ammonium ions.

EFFECT OF L- AND D-ASPARTATE ON THE UPTAKE AND UTILIZATION OF AMMONIUM-N: Table I indicates that the presence of L-aspartate in the external medium accelerated the uptake of ammonium-N, while on the other hand, D-aspartate retarded ammonium absorption. Moreover, table II shows that when D-aspartate was added to L-aspartate-ammonium sulfate media, the stimulating effect of L-aspartate on the

TABLE III

EFFECT OF CULTURING CARROT ROOT DISKS FOR 24 HOURS IN SOLUTIONS OF 0.0025 M L-, D-, OR DL- AMMONIUM ASPARTATE AND ASPARAGINE

NITROGEN FRACTIONS	MG N/100 GM FRESH WT OF TISSUE *					
	CONTROL IN DIST. H_2O	CHANGES IN THE N FRACTIONS AS COMPARED WITH THE CONTROL				
		AMMONIUM ASPARTATE			ASPARAGINE	
		L-	D-	DL-	L-	D-	DL-
Ammonium-N	0.88	+ 6.73	+ 9.06	+ 7.52	- 0.19	+ 0.88	+ 0.36
Glutamine-N	6.66	+47.30	+15.63	+38.60	+ 5.74	+ 7.01	+ 5.69
Asparagine-N	5.95	+ 9.31	+ 4.52	+ 1.96	+85.00	+81.40	+85.10
Amino acid-N	11.86	- 2.29	+ 6.18	+ 0.88	- 0.99	- 0.80	- 3.37
Rest-N	12.15	+ 1.13	+ 1.39	+ 3.62	+ 2.41	+ 0.99	+ 5.89
Protein-N	89.40	+26.10	+ 3.10	+16.95	+ 6.64	+ 1.41	+ 4.60
Absorbed nitrogen							
Ammonium-N	....	55.5	28.50	45.1	....	....	....
Amide-N	....	....	....	....	49.6	45.9	49.1
Amino-N	....	30.2	10.35	23.9	49.9	44.6	48.3
Utilized nitrogen							
Ammonium-N	....	49.4	20.10	38.3	....	....	....
Amino acid-N	....	32.3	3.98	22.9	....	....	....
Asparagine-N	....	....	....	....	13.44	9.62	12.30

* Means of duplicate samples.

uptake of ammonium-N was neutralized by the retarding effect of the D-isomer. Similarly the absolute amounts of ammonium-N removed from the medium containing DL-aspartate plus ammonium sulfate approached that removed from the solution containing ammonium sulfate alone. It is thus again demonstrated that D-aspartate, the absorption of which lagged very much behind that of L-aspartate, exerted a depressing effect on the uptake of ammonium-N. A more or less similar phenomenon was demonstrated by El-Shishiny and Nosseir (3) with glutamate isomerides.

L-Aspartate in 0.0025 M concentration stimulated both the absorption and assimilation of ammonium-N. When the concentration of L-aspartate was increased to 0.005 M, the increase in uptake of ammonium-N was doubled while that of its assimilation was increased 5-fold. On the other hand, addition of D-aspartate to ammonium sulfate in the culture solution reduced the amount of ammonium-N assimilated by carrot root disks, probably on account of a similar effect on its absorption.

EFFECT OF L- AND D-ASPARTATE AND ASPARAGINE ON AMIDE FORMATION IN CARROT ROOT DISKS: The results obtained from culturing carrot root disks in solutions of L-aspartate alone and in combination with ammonium sulfate (tables I and II) indicated the utilization of aspartic acid in the primary formation of the amides. This was inferred from the great increase in the amide-N × 2 while the other more complex nitrogenous compounds showed a positive balance. On the other hand, the smaller increase in amide-N × 2 recorded in carrot disks cultured in D-aspartate alone might be at the expense of rest-N which showed a more or less equal decrease. This conclusion was substantiated by the results in table I, since carrot root disks cultured in D-aspartate together with ammonium sulfate showed no increase in the levels of the organic nitrogenous fractions, other than amino acids, over those of control tissue in ammonium sulfate alone. The increase in the amino acid-N levels was roughly equal to the amount absorbed. Furthermore, the results in table II did not show an increase in amide-N content of carrot disks cultured in solutions containing D- + L- or DL-aspartate together with ammonium sulfate.

The considerable amounts of primary amides formed in carrot root disks cultured in ammonium sulfate alone or in combination with L-aspartate were mainly of glutamine. Asparagine contributed a smaller fraction of the amides formed. The accumulation of primary glutamine in carrot root cells even when L-aspartate forms the nitrogen source, might be an evidence that aspartic acid is a very active compound, being converted into glutamic acid and glutamine. This explains why asparagine did not appreciably increase in tissues supplied with ammonia alone, although aspartic acid might have been abundantly formed at first. Similar results were obtained from the pea experiments by Rautanen (5). The primary formation of glutamine when aspartic acid formed the nitrogen source to carrot root disks might also suggest the transference of the amino group of aspartic acid to α-ketoglutaric acid abundantly produced through the respiratory cycle (1), and the subsequent amidation of glutamic acid. This suggestion gained support from the finding of Leonard and Burris (4) that carrot extract possessed a high transamination rate towards glutamic acid production from aspartic acid and α-ketoglutaric acid. The existence

of enzyme systems responsible for the coupling of ammonia and aspartic acid was shown by Webster and Varner (11), in lupine and in wheat germ tissues.

In conjunction with the evidence already discussed, the accumulation of asparagine in carrot root tissues cultured in asparagine solutions (table III) did not speak in favor of possible direct transamination or transamidation reactions between asparagine and α-ketoglutaric or glutamic acid.

Summary

The uptake and utilization of the stereoisomers of aspartic acid and asparagine as well as the effect of the formers on the uptake and utilization of ammonium-N by carrot root disks were investigated.

1. Absorption of L-aspartate is much faster than that of its antipode. The amount of D-aspartate absorbed during 24 hours was less than 50 % of that of L-aspartate, when they were externally supplied, alone or in combination with ammonium sulfate.

2. L-Aspartate enhanced the absorption and utilization of ammonium-N, but D-aspartate depressed both processes.

3. L-Aspartate was markedly utilized causing a greater increase in the protein than in amides when supplied alone, but more amides than protein when supplied in combination with ammonium sulfate. Aspartic acid was rapidly assimilated forming liberally glutamine; asparagine was formed, however, at a lower rate. On the other hand, D-aspartate taken up by carrot root disks behaved as an inert metabolite.

4. Asparagine was taken up by carrot root cells as intact molecules. Almost all asparagine absorbed accumulated as such in the cells.

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RUBBER BIOSYNTHESIS IN LATEX OF HEVEA BRASILIENSIS[1,2,3]

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The ubiquitous occurrence and economic importance of the isoprenoids has stimulated a great amount of research on the mechanism of their synthesis by plants (6, 8). Arreguin et al (1, 2) and Bonner and Arreguin (7) studying rubber synthesis in seedlings and tissue slices of guayule (*Parthenium argentatum*) have established that rubber is derived from acetate and that β-methylcrotonate is a probable intermediate between acetate and the polymerized isoprenoid. Considerable progress has also been made towards understanding the mechanism of biosynthesis of the branched chain 5 and 6 carbon acids which serve as rubber precursors (4, 5, 6, 9, 14, 17, 18, 19). A further elucidation of the metabolic pathway of rubber synthesis and especially of the steps leading to formation of the long chain polymer has been hampered by the necessity of utilizing intact plants or tissue slices as experimental material. We wish to report here the first in vitro synthesis of rubber. An enzyme preparation from latex of the commercial rubber tree, *Hevea brasiliensis* has been shown to catalyze the incorporation of C^{14}-labeled acetate into

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rubber thus indicating the presence in latex of all of the enzymes necessary to form rubber from acetate. Cell-free systems capable of synthesizing cholesterol from acetate have previously been described (10, 12). Since, however, the rubber hydrocarbon consists of a linear chain of isoprenoid units, without cyclization such as occurs in steroids, it is believed that the presently described system will prove of value in studies of the detailed mechanism of isoprenoid biosynthesis. A preliminary report of this work has been published (24).

Materials and Methods

Latex was obtained from a seedling tree (approximately 50 centimeters in diameter) which had been recently brought into tap. Aliquots of freshly collected latex were pipetted into the indicated incubation media contained in 3-ml centrifuge tubes, mixed, and incubated at 37° C. Incubation was terminated by coagulating the latex with five volumes of acetone. After the coagulated latex had been standing several hours at room temperature the acetone was decanted, the plugs of latex transferred to paper thimbles, and the thimbles soaked 8 hours, with one change of solution, in 25 % aqueous acetone containing 1 % acetic acid. The thimbles were next extracted with acetone in a Soxhlet apparatus for two hours to dissolve resins and other non-rubber materials. Following acetone extraction the thimbles were air dried, then (to facilitate solution of the rubber) immersed in benzene containing 1 % trichloroacetic acid, and heated at 60° C for two hours with intermittent agitation. The thimbles were next transferred to a Soxhlet apparatus and extracted for two hours with benzene. The combined benzene extracts were brominated by the method of Gowans and Clark (13) and the fine precipitate of rubber bromide washed three times with 95 % ethanol. The washed rubber bromide samples were either dried as a slurry in planchets or collected on filter paper discs by the use of a filtration apparatus. Samples of rubber bromide were counted by means of a gas flow proportional counter. All figures presented have been corrected for self-absorption and represent the average of duplicate determinations.

Table I

Time Course of Incorporation of Acetate into Rubber

Incubation time	Total counts incorporated
hrs	*cpm/tube*
0	155
2	1680
4	2130
8	2680

Each tube contained in micromoles: adenosine triphosphate 1, magnesium fructose diphosphate 2, coenzyme A 0.01, diphosphopyridine nucleotide 0.01, ethylenediaminetetraacetate 1, potassium phosphate 1, sucrose 60, sodium acetate-1-C^{14} (containing approximately 5×10^6 cpm) 2, and latex 0.1 ml. Total volume 0.3 ml, pH 7. Incubation was at 37° C for the indicated time.

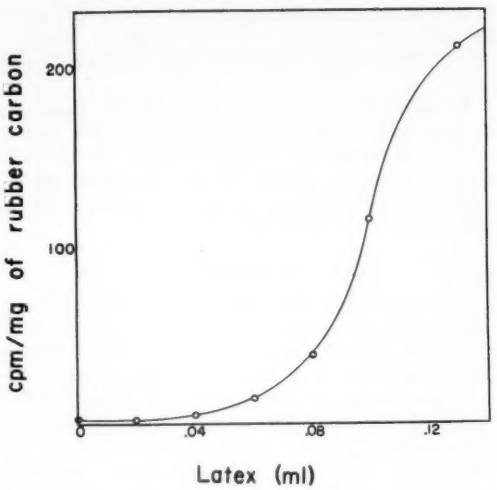

Fig. 1. Dependency of acetate incorporation into rubber upon concentration of latex in incubation mixture. Addenda as for table I. Incubation time 4 hrs at 37° C.

Experimental

In preliminary experiments it was established that C^{14}-acetate was indeed incorporated into rubber by the latex enzyme system and that a cofactor mixture increased the incorporation. A time course for the reaction is shown in table I. The continued incorporation past four hours indicates a relatively stable enzyme system. Although no protein determinations were made it can be calculated from published data (8) that the activity in 0.1 ml of latex may be attributed to one mg or less of protein.

The small incorporation observed at zero reaction time is probably owing to the necessary manipulation time and to the time lag between addition of acetone and final coagulation of the inside of the latex plug. That such "time zero" counts do not represent contaminating acetate was determined by an experiment in which latex was pipetted into radioacetate and acetone. In this case no radioactivity was found in the isolated rubber bromide.

The effect of enzyme concentration on the rate of acetate incorporation into rubber is shown in figure 1. As can be seen activity is not directly proportional to enzyme concentration suggesting that at lower enzyme concentrations incorporation may be limited by some readily dissociable cofactor.

The effect of various additions to the reaction mixture on the rate of acetate incorporation into rubber is shown by the data of table II. Addition of sucrose alone had no effect; the cofactor mixture alone caused about a four-fold increase; while addition of sucrose and cofactors together increased incorporation to over ten times that of the unsupplemented latex. Although

TABLE II

EFFECT OF SUCROSE AND COFACTORS ON ACETATE INCORPORATION INTO RUBBER

ADDITIONS	INCORPORATION INTO RUBBER
	cpm/mg of rubber carbon
Basic *	26
Basic + sucrose **	13
Basic + cofactor mixture **	91
Basic + sucrose + cofactor mixture **	297

* The basic reaction mixture contained 0.1 ml latex, 2 micromoles of sodium acetate-1-C^{14}, and distilled water to 0.3 ml.

** Additions of sucrose and cofactor mixture at the same concentration as for table I. The cofactor mixture consisted of adenosine triphosphate, magnesium fructose diphosphate, coenzyme A, diphosphopyridine nucleotide, ethylenediaminetetraacetate, and potassium phosphate. Incubation was for 4 hours.

these limited results do not permit a decision as to which of the added cofactors were required, they do establish that the latex enzyme system will be amenable to a determination of the cofactors required for the conversion of acetate into rubber.

Several C^{14}-labeled substrates other than acetate were tested for their ability to serve as a source of carbon for rubber synthesis. The results of experiments with sucrose, pyruvate, β-methylcrotonate and carbon dioxide (as potassium bicarbonate) are shown in table III. As can be seen acetate was most effective, pyruvate about two thirds as effective and β-methylcrotonate about one third as effective as acetate. Neither carbon dioxide nor sucrose was incorporated.

TABLE III

INCORPORATION OF SUBSTRATES OTHER THAN ACETATE INTO RUBBER

SUBSTRATE *	SPECIFIC ACTIV. SUBSTR.	INCORPORATION **	RELATIVE EFFICIENCY
	cpm/micromole	*cpm/mg of rubber carbon*	*%*
Sucrose	7.0×10^4	0	0
$KHC^{14}O_3$	7.5×10^5	0	0
Sodium-3-C^{14}-β-methylcrotonate	1.8×10^5	11	36
Sodium-2-C^{14}-pyruvate	9.1×10^5	42	60
Sodium-1-C^{14}-acetate †	2.5×10^6	207	100

* All preparations contained cofactors and latex at the concentrations used for table I. Unlabeled sucrose was added to all tubes except that in which C^{14}-sucrose served as substrate. The following amounts (in micromoles) of labeled substrates were employed per tube: sucrose 60, $KHCO_3$ 20, β-methylcrotonate 4, pyruvate 1.9, and acetate 2. Incubation was for 4 hrs.

** Incorporation is given as observed counts minus the respective time zero control counts.

† The value for acetate represents an average value of several experiments conducted under the same conditions.

An attempt was made to fractionate the latex enzyme by centrifugation. For this purpose fresh latex was centrifuged for fifteen minutes in a chilled centrifuge head at a relative centrifugal force of $15{,}000 \times g$. As has been previously reported (11) a separation was obtained into three main fractions; a top layer of white, pasty rubber; a grayish white serum layer, containing some rubber; and a precipitate of yellow sediment called "yellow fraction." The speed of centrifugation was such that mitochondria should have been sedimented into the yellow fraction. However the high rubber content of latex (35 to 40 %) undoubtedly interfered with normal particle sedimentation. The relative activities of the various fractions are shown in table IV. As can be seen the serum was most active, the increase in activity over whole latex being approximately the amount to be expected from removal of the rubber. It is clear that yellow fraction resuspended in serum was not as active as serum alone, and thus, particles as large as mitochondria are probably not involved in rubber synthesis. The possibility that microsomes may be involved has not

TABLE IV

INCORPORATION OF ACETATE INTO RUBBER BY LATEX FRACTIONS

FRACTION *	VOLUME	INCORPORATION
	ml	*cpm/tube*
Whole latex	0.1	4940
Serum	0.1	7860
Yellow fraction re-suspended in serum	0.1	3360

* All tubes contained the addenda listed for table I except for latex which was as shown above. Incubation was for 4 hrs.

been eliminated, since the centrifugal forces employed would not have sedimented microsomes.

Since the experiments reported above were all performed on latex derived from a single tree, additional experiments were done using latex from 33 other trees of varying age and yield growing in Puerto Rico and Liberia. All lactices tested were active in catalyzing the incorporation of acetate into rubber. These results will be the subject of a separate report.

In the present experiments the material termed "rubber" was characterized by its solubility and the solubility of its bromide derivative. It seemed desirable however to establish by an independent method that the acetate carbon had in fact been incorporated into rubber. Accordingly the specific activities of rubber isolated as rubber, and as the rubber bromide were compared with that of levulinic acid 2,4-dinitrophenylhydrazone prepared by ozonolysis of the rubber. The ozonolysis procedure has previously been shown to yield levulinic acid in good yield (16).

Rubber for ozonolysis was prepared by incubating 1.4 ml of latex with 14 times the amount of sucrose and cofactors indicated in the legend of table I for

four hours. The plug of coagulated latex was extracted with acidified aqueous acetone and then for two hours with acetone in a Soxhlet apparatus. The latex coagulum was next cut into very thin slices, extracted an additional hour with acetone, and dried. The rubber was dissolved by treatment with 30 ml of boiling benzene, then heated at 65 to 70° C for three hours with intermittent swirling, and allowed to stand overnight. The rubber solution was filtered, aliquots taken for bromination, and the remaining rubber precipitated by the addition of ten volumes of absolute ethanol. After standing overnight, the thin film of precipitated rubber was rinsed with ethanol, dried, dissolved in carbon tetrachloride, and aliquots taken for bromination. Glacial acetic acid was next added, and the rubber ozonized at 0° C for 130 minutes. The solution was then warmed, the carbon tetrachloride distilled off under vacuum, and the glacial acetic acid solution of ozonides hydrolyzed by the use of water and 30 % H_2O_2 (21). When paper chromatography of the 2,4-dinitrophenylhydrazone derivatives of small aliquots showed the predominant product to have the R_f of the levulinic acid derivative, the main batch was treated with 2,4-dinitrophenylhydrazine, the derivative crystallized from ethanol, and purified through the sodium salt (21). The material so prepared was found by paper chromatography to be free of other 2,4-dinitrophenylhydrazones.

The average radioactivity of the rubber bromide from the benzene solution of rubber, the rubber bromide from the carbon tetrachloride solution of alcohol precipitated rubber, and the levulinic acid 2,4-dinitrophenylhydrazone derivative were, respectively, 219, 198, and 227 cpm/mg of rubber carbon. The correspondence of the counts in the rubber and levulinic acid provides strong evidence that the acetate had indeed been incorporated into rubber.

Discussion

In most of the experiments here reported a mixture of sucrose and cofactors was added together with latex and C^{14}-labeled acetate to obtain maximal rubber synthesis. The data of table II show however that latex without supplements other than acetate synthesizes rubber. Thus, these experiments establish the latex as a site of rubber synthesis in vivo, and are in accord with previous suggestions that rubber synthesis occurs within the lactiferous elements (cf 8, page 550). It would seem reasonable however to anticipate a greater concentration of rubber synthesizing enzymes in the cytoplasmic layer which remains in the lactiferous vessels during tapping.

No conclusions may be drawn as to which of the materials present in the cofactor mixture (adenosine triphosphate, magnesium, fructose diphosphate, coenzyme A, diphosphopyridine nucleotide and potas-

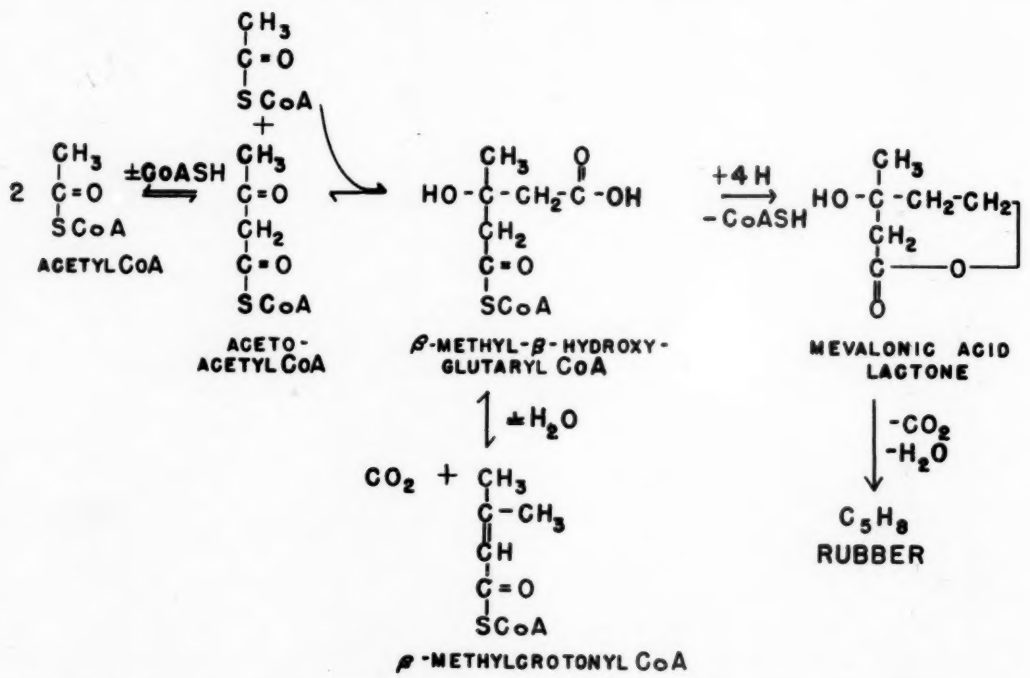

Fig. 2. A possible mechanism for the biosynthesis of rubber modified after Bonner (6). Incorporation of acetoacetate, β-methylcrotonate and β-methyl-β-hydroxyglutarate into rubber in a system synthesizing rubber from acetate would depend upon the presence of the appropriate, "activating," enzyme.

sium phosphate) caused the observed stimulation of rubber synthesis. It would not be unexpected to find adenosine triphosphate and coenzyme A to be required since the role of these compounds in acetate activation is known (15). The stimulation of rubber synthesis by sucrose may be owing to the requirement by the system for reducing power, although fructose diphosphate should have played that role.

Of the various substrates tested for incorporation into rubber, acetate proved best. Sucrose and CO_2 were not incorporated while pyruvate and β-methylcrotonate were less effective than acetate. The incorporation of pyruvate is not surprising in view of its ability to serve as an acetyl source for acetyl–coenzyme A formation. The ability of β-methylcrotonate to serve as substrate would indicate the presence of an activating system in latex if indeed β-methylcrotonate reacts as β-methylcrotonyl-coenzyme A. The failure of sucrose to be incorporated despite the stimulation by sucrose of rubber synthesis from acetate, may indicate a slow rate of glycolysis of sucrose in latex. Hevea tissue slices have been observed to incorporate labeled sucrose into rubber (Teas, unpublished).

The failure of $C^{14}O_2$ to become incorporated into rubber is most readily explained by the asymmetry of β-methyl-β-hydroxyglutarate as the mono-coenzyme A ester. Thus even though β-methyl-β-hydroxyglutaryl-CoA may be reversibly decarboxylated to form a C_5 acid and CO_2 (3), the same carboxyl might be lost during the ultimate polymerization reaction. Johnston, Racusen and Bonner (14) have observed the incorporation of radioactivity into β-methylcrotonate, β-methyl-β-hydroxyglutarate and acetoacetate by a flax enzyme system incubated with β-methylcrotonate and $C^{14}O_2$. They postulate equilibration of the free carboxyl of β-methyl-β-hydroxyglutarate with the acyl-CoA carboxyl by a side reaction involving cleavage and resynthesis of β-methyl-β-hydroxyglutaryl-CoA. This equilibration apparently does not occur in the latex system. The recent studies of Tavormina (22, 23) while establishing a role for mevalonic acid clearly do not exclude β-methylcrotonate, or β-methyl-β-hydroxyglutarate as intermediates in terpene synthesis (See below). A biosynthetic pathway, modified slightly from those previously described (5, 6, 14, 15), which accounts for the failure of $C^{14}O_2$ to be incorporated into rubber and which includes the recent observations of Tavormina et al (22), is presented in figure 2.

If as here indicated, acetate, acetoacetate, β-methylcrotonate, and β-methyl-β-hydroxyglutarate are all active as the acyl–coenzyme A compounds, the rates of incorporation of each of these compounds into rubber may depend upon the relative activity of the appropriate activating system. In experiments comparing the rates of incorporation of the free acids into rubber one may not conclude from the relative activity of the acid, or even from its failure to be incorporated, that it is or is not a direct precursor. Thus it is possible, as studies on fatty acid metabolism (20) have amply demonstrated, for the free acid to be totally inactive whereas the coenzyme A derivative is rapidly metabolized.

Summary

1. Hevea latex has been demonstrated to catalyze the incorporation of C^{14}-labeled acetate into rubber in vitro.

2. The incorporation of acetate into rubber is stimulated by the addition of sucrose and a cofactor mixture containing adenosine triphosphate, coenzyme A, diphosphopyridine nucleotide, magnesium ion, fructose diphosphate and potassium phosphate.

3. The enzyme system responsible for conversion of acetate into rubber remains in the supernatant fluid following centrifugation of latex at $15{,}000 \times g$.

4. C^{14}-labeled pyruvate and β-methylcrotonate are incorporated into rubber by the latex enzyme system whereas sucrose and $C^{14}O_2$ are not.

5. That acetate is in fact incorporated into rubber has been demonstrated by a comparison of the specific activities of rubber isolated as rubber and as the bromide with that of levulinic 2,4-dinitrophenylhydrazone prepared by ozonolysis of the biosynthesized rubber.

6. The advantages of the latex system for studies of the biosynthesis of the terpenoids are discussed.

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RELATIONSHIP OF BORON NUTRITION TO RADIOSENSITIVITY OF SUNFLOWER PLANTS[1,2]

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The absence of boron for even short intervals results in retardation of cellular activity and arrested growth in plants. Since radiosensitivity is related to cellular and metabolic activity, some relationship might be expected to exist between radiosensitivity and boron nutrition, and the use of ionizing radiations might be expected to prove useful for the study of the physiological role of boron and perhaps of other essential elements. Experiments designed to test for the presence of such a relationship have indicated that it does in fact exist (8). These experiments and their indications pertaining to the role of boron in the plant cell will be described fully here.

Materials and Methods

Mammoth Russian sunflower plants were grown in nutrient solutions in a controlled environment growth room under conditions previously described (9). The seed were sown in quartz sand and watered with tap water. The seedlings were used one week from sowing at which time they had expanded cotyledons but no emerged leaves. They were transferred to nutrient solutions with 0.5 ppm of boron, or without boron, contained in wide-mouth, low-boron, soft glass quart fruit jars wrapped in black cloth to exclude light. The plants were supported by slotted stainless steel holders and held in place by a pliable asbestos mastic (Kalk-Kord). The responses of sunflower to absence of boron under these cultural conditions have been described (9).

The irradiations were carried out with a Westinghouse 200-KV Duocondex x-ray machine, operated at 200 KV and 15 milliamperes with no added filtration. The entire top portions of the plants were irradiated but the roots were shielded with 2-inch lead bricks. The plants were placed at distances varying in different experiments from about 21 to 27 inches from the x-ray source and the delivered dose rates were between 40 and 68 roentgens (r) per minute. Dose rates were determined by a Victoreen dosimeter.

The plants were generally irradiated on the third day from time of transferring to the nutrient solutions (after about a 72-hour growth period in the solutions), although some irradiations were made on the second, fourth, and fifth days.

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After x-irradiation all plants were always supplied with available boron regardless of their previous treatment. They were either transferred to + B nutrient solutions for a few days and then transplanted to soil or transplanted to soil directly. The ensuing responses were identical under either procedure. The plants were maintained in the controlled environment growth room for several weeks and were then transferred to the greenhouse if they were to be maintained for extended periods.

Results

Early Seedling Development in Response to Boron: Sunflower seedlings were examined under a dissecting microscope at various stages of growth. The embryo of the seed contains two nodes, the cotyledonary node and the primordia of the first pair of leaves. The primordia of the first pair of leaves in the dry seed consists of two protuberances placed side by side and so close together that they almost appear as a single parabaloidal structure. One day (24 hours) after sowing, the two primordial leaves have become slightly enlarged and elongated and may be separated with a dissecting needle thus showing a flat meristem. By the second day after sowing the primordia of the first pair of leaves have enlarged further and have become partially separated. On further separation with the dissecting needle the flat meristem may again be seen, with, as yet, complete absence of primordia of the second pair of leaves. By the third day the primordia of the second pair of leaves (at the 3rd node) are noticeable as two very small bulges, opposite from each other on the perimeter of the flat meristem and enclosed by the primordia of the first pair of leaves. By the fourth day the first pair of leaves has enlarged further and has become slightly green and slightly pubescent (young leaves at later stages have many hairs). The primordia of the second pair of leaves are still small but are now closer together, hiding the meristem. Their removal or separation reveals the flat meristem which shows no evidence of primordia of the third leaf pair. The primordia of the third pair of leaves are generally visible by the fifth day and are well developed by the seventh day.

The primordia of the leaf pairs are generally equal in size in case of the first and second pair (at the second and third nodes), but starting with the third pair (at the fourth node) one primordial leaf is generally larger than its partner.

The bud of a more mature plant at any particular time contains about six nodes if only the very immature leaves which clasp and enclose the bud are included. The bud is slender and elongated but the meristem remains flat.

At the 7th day from sowing which is the time the seedlings were transferred to the nutrient solutions at the start of the experiments, the seedlings had four nodes–the cotyledonary node and three pairs of leaves, but none of the leaves had emerged from the terminal bud at this time (fig 1). Three days (about 72 hours) later, if grown in + B nutrient solutions during this period, the plants had seven nodes; the first pair of leaves was partially emerged, and each was about 1 to 2 cm long. Plants of the same age but grown in – B solutions for the 3-day period had six nodes, and they were considerably compacted in comparison with the structures in the plants grown in + B solutions. However, observations with a dissection microscope revealed no necrosis or visible tissue damage. The first pair of partially emerged leaves was somewhat smaller, on the average, than that of the + B plants,

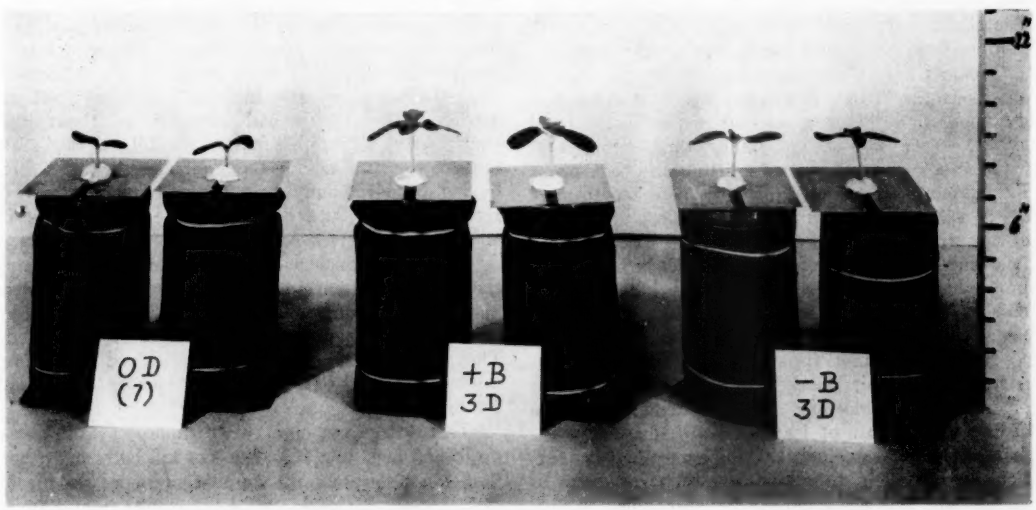

Fig. 1. Early stages of development of sunflower plants in nutrient solutions. *Left,* seedlings at time of transference to solutions, 7 days after sowing seed in sand (start of experiment); *center,* seedlings after 3 days in + B solutions; *right,* seedlings after 3 days in – B solutions.

but the plants had not yet shown distinct or recognizable gross boron deficiency symptoms (fig 1). The compactness of the nodes, the production of six nodes instead of seven, and the smaller size of the first pair of leaves indicate, however, that growth had been measurably retarded during this 3-day growth period; these early morphological changes precede the characteristic boron deficiency symptoms that subsequently develop (9).

Responses to X-Irradiation: Plants grown under normal nutritional conditions were irradiated at various roentgen dosages to determine the type of irradiation responses produced and the relative radiosensitivity of this species. They were irradiated on the third day after transferring to + B solutions (10 days from sowing) at which time they had produced seven nodes but had only their first pair of leaves partially expanded. After irradiation the plants were treated in the usual manner and were permitted to grow for observation of rate of development and appearance of subsequent tissues. Responses to seven different dosages ranging from 100 to 5000 r are described.

100 r: No observable effects of any sort were apparent.

500 r: Plants appeared normal up to the fourth or fifth day following irradiation. On the sixth day the first pair of leaves were normal but a slight mottling appeared in the second pair of leaves and later also appeared in the third and fourth pair as these structures developed. These leaves, however, were of normal size and shape, exhibiting no distortion or wrinkling. The height of the plants at no time differed from that of unirradiated control plants. The fifth pair of leaves, which expanded at about the 16th day after irradiation, were again normal and free of any mottling and all subsequent growth was normal and indistinguishable from that of control plants. The slight mottling of the second, third, and fourth pairs of leaves persisted during this time.

750 r: Characteristic mottling of leaves developed on about the fourth day following irradiation, and was much more severe than that in plants irradiated at 500 r. In addition to leaf mottling considerable leaf distortion, serration and splitting also took place. Furthermore, this irradiation effect persisted in most of the subsequently formed leaves almost up to time of flowering. Some plants maintained until 82 days following irradiation grew up to six feet in height and produced distorted leaves up to the 26th or 27th node position. The height of the plants was not affected by this dose.

1000 r: The first apparent symptoms, which occurred on the fourth day after irradiation, consisted of characteristic mottling as well as reduction in size of the second pair of leaves. Later, generally, the first pair also became mottled. The mottling was particularly severe in the third and following pairs of leaves, and was accompanied by pronounced distortion, wrinkling, splitting, and abnormal marginal serration of leaves. These symptoms persisted in all leaves produced up to about the 28th to 30th node, at which time flowering occurred, in plants maintained for 82 days; and they were even more severe in the last formed leaves than in comparable leaves of the 750 r plants. The buds of plants receiving 1000 r were not killed and the plants produced the same number of nodes as did the control plants. Some stunting occurred although the total height was only slightly reduced in some plants and not at all in others; reduction in growth was primarily manifested in reduction of leaf size and general plant vigor.

1500 r: Plants exposed to this dosage developed radiation symptoms similar but considerably more severe than those exposed to 1000 r. The plants were fasciated, distorted, and considerably stunted. Definite reductions in height as well as in node number occurred.

2000 r: The first noticeable radiation symptoms in this lot also appeared on the fourth day after irradiation as mottling and reduction in size of the second pair of leaves. A few days later the first pair of leaves became slightly bleached in some areas and the second pair were extremely mottled, bleached, and curled. The third pair was extremely distorted when it developed and by about the 12th to 13th day after irradiation dead and dry areas appeared on most leaves. By this time, the plants were very stunted. These plants produced a total of only four pairs of emerged leaves, after which all growth stopped. At the 18th day a considerable portion of leaf tissue was dead and by the 23rd day most of the top portion, including the terminal buds in most of the plants, had been killed.

5000 r: On the fourth day following irradiation, the second pair of leaves was reduced in size and mottled, and, shortly thereafter they became bleached. The first pair became buckled and bleached, and, by about the 10th to 12th day after irradiation, both pairs of leaves were curled, very bleached and had many dead and dry areas. The third pair of leaves was just visible as two small points on about the 9th day; they were severely bleached, and developed no further. All growth had stopped at about the twelfth day; by approximately the 15th to the 18th day the tips and most leaf tissue had been killed after having produced only two pairs of emerged leaves. By the 20th to 23rd day, the entire top portions of all the plants were dead.

The roots, which were shielded during irradiation, showed no effects of any sort in any of the irradiated plants. They appeared to be able to make normal growth even though top growth was severely affected. They even retained a normal appearance for a considerable period after the top portions were killed at the higher doses.

This dosage response survey indicates that doses up to and including 1000 r do not kill buds and other tissues nor decrease the number of nodes produced. Only a slight depression of height results from 1000 r, although marked foliar radiation symptoms are produced. The higher doses, particularly 2000 and 5000 r, are very damaging and result in death of the ir-

radiated parts. These responses further indicate that the sunflower plant is relatively very radiosensitive when compared with numerous other plant species (11).

The dose of 1000 r, which does not kill but produces marked and persistent radiation effects, was found to be the most useful dose for the purposes of the present experiments. Responses to 500 r are temporary and the higher doses are excessively damaging.

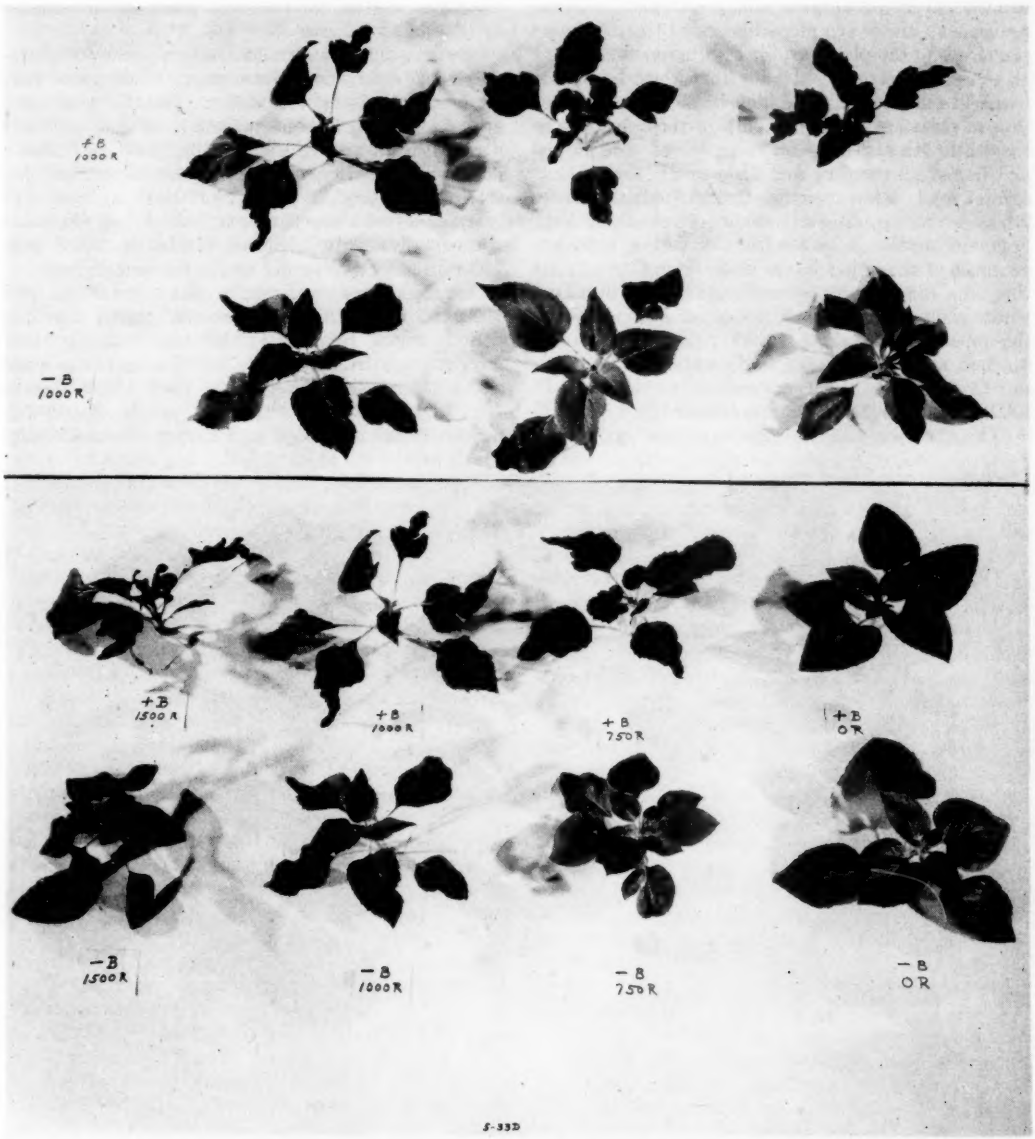

FIG. 2 (*above*). Top view of sunflower plants 33 days after x-irradiation at 1000 r. Top row, plants not deprived of B at any time; bottom row, plants deprived of B for 3 days prior to irradiation. (Plants were cut at 6th node and placed in flasks over which were placed a sheet of white background paper for purposes of obtaining photograph of tips and most recently formed leaves.)

FIG. 3 (*below*). Top view of sunflower plants 33 days after x-irradiation at, left to right, 1500, 1000, 750, and 0 r. Top row, plants not deprived of B at any time; bottom row, plants deprived of B for 3 days prior to irradiation. (Plants arranged for photography as in fig 2.)

Effect of Boron Availability on Irradiation Responses: Plants were irradiated with 1000 r after a 3-day (72-hour) growth period in + B or − B nutrient solutions (fig 1). Boron was then made available to all plants immediately after irradiation. Typical radiation symptoms developed in the subsequently formed leaves in both groups of plants, and, as stated previously, these symptoms persisted in all leaves produced by the plants grown with boron available at all times. However, the plants from which boron was withheld for three days prior to irradiation developed similar radiation symptoms only in the leaves up to the 6th to 7th node; the following leaves were normal and free of all mottling and distortion. These plants several weeks later appeared normal from the 6th to 7th nodes on up but the lower leaves maintained the original symptoms. This marked difference in the appearance of the upper leaves is shown in figure 2. At this time (33 days after irradiation) the + B, 1000-r plants show marked distortion of all leaves whereas the upper leaves of the − B, 1000-r plants are normal. Marked radiation-induced malformation of leaves at the 18th to 28th nodes was still evident in the + B, 1000-r plants 66 days after irradiation (fig 4, left).

These results, which were duplicated in several separate experiments, clearly indicate an intimate relationship between boron nutrition and radiosensitivity. Apparently a reduced rate of cellular activity brought about by temporarily withholding boron results in greater radioresistance. The radiation responses of a plant are apparently dependent, in part, upon the kind of physiological processes in progress at time of irradiation.

Similar differential radiation symptoms were produced by withholding boron using other doses, particularly with 750 r and 1500 r. The effects of 500 r are temporary and slight, making it difficult to clearly distinguish alleviating effects. The 2000 and 5000 r doses are excessively damaging; the plants receiving these doses produce only four and two pairs of emerged leaves respectively, and alleviating effects do not occur so early. Normal plants irradiated with 750 r produce symptoms similar to but slightly less severe than those occurring in plants irradiated with 1000 r. The symptoms, however, persist for long periods (fig 4, right). Withholding boron for three days prior to irradiation at 750 r produces the usual alleviation of symptoms in the later formed leaves (fig 3), also starting with those at the 6th to 7th nodes. Plants irradiated with 1500 r are considerably

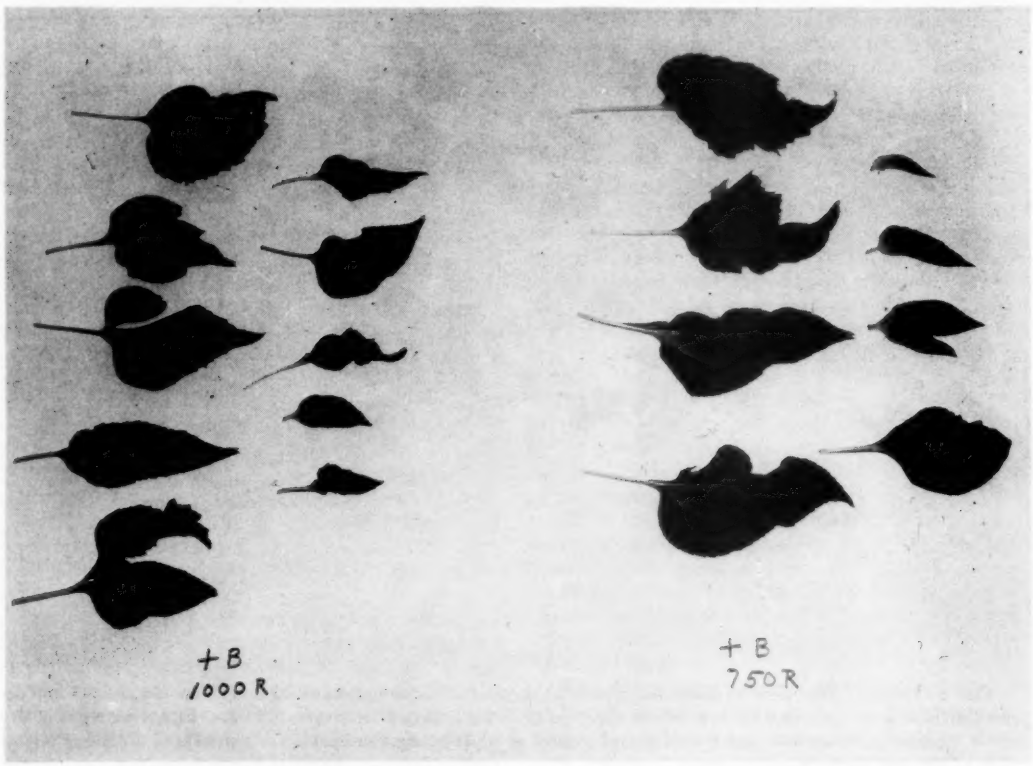

Fig. 4. Upper leaves of sunflower plants not deprived of B at any time, taken from 18th to 28th nodes 66 days after irradiation at 1000 r, left and 750 r, right. (Plants similarly irradiated but deprived of B for 3 days preceding irradiation produced normal leaves starting at about the 7th node.)

more damaged than those receiving 1000 r, and radiation damage is even noticeable in the upper leaves of the – B, 1500-r plants, although definite alleviation is also produced (fig 3) by withholding of boron for three days prior to irradiation. The upper leaves of the – B, 1500-r plants are relatively free of marked distortion but they are generally smaller in size in comparison to those of O-r or – B, 1000-r plants.

Differential irradiation symptoms were also produced in experiments in which boron was withheld for periods other than three days prior to irradiation, although the 3-day period was found to be most satisfactory. If boron was withheld for two days prior to irradiation, differences between + B and – B plants irradiated with 1000 r appeared later (beyond the usual 6th to 7th node positions) and were slighter and less distinct than those obtained with the 3-day withholding period. With a 4-day withholding period, the differences were definite but the – B plants required a longer time to recover from the boron deficiency; after recovery had occurred, the upper portions of the – B, 1000-r plants were normal while the + B, 1000-r plants showed the typical persistent symptoms. With a 5-day withholding period, the plants require an even longer period to recover from boron deficiency and it is difficult to distinguish clearly between boron-deficiency malforamation and radiation-induced malformation. A clear demonstration of the relationship of boron nutrition to irradiation responses would undoubtedly depend on the experimental conditions employed which would include such factors as the degree of purity of the boron deficient solutions and perhaps factors such as temperature and light conditions which would in turn influence growth rate.

Relative Radiosensitivity of Growth Phases: The results of experiments on the effect of boron availability on radiation responses indicate that the temporary withholding of boron prior to irradiation greatly alleviates irradiation damage. Withholding boron slows down some cellular process or processes thus making the cells more radioresistant. This phase of cellular activity then, must be specifically dependent upon boron; it may be cell division or cellular differentiation or even possibly differentiation of a particular kind. If this radiosensitive phase of cellular activity could be identified, it would contribute to the elucidation of the role of boron in the plant cell. Experiments were designed to examine the relative radiosensitivity of growth phases in the developing seedling.

Sunflower seed were first soaked in water for 15 minutes and then placed on moist filter paper in covered dishes, water being applied as needed, and allowed to germinate at 75° F. Samples were taken at various intervals (20, 25, 42, 49, 66, 73, 90, and 97 hours from time seed had started to soak) and were x-irradiated with 1000 r and planted. Ten plants of each lot, along with nonirradiated controls for successive plantings, were maintained for periods up to eight weeks for the observation of relative radiation effects. Plants irradiated at various intervals will be identified as to time of irradiation following the soak period (e.g., n-hour irradiated plants); the time of observation will also be referred to the start of the soak period (e.g., at the nth day).

Plants photographed on the 18th day, showing differential radiation response to 1000 r delivered at various stages of germination, are shown in figure 5. On the 15th day the first pair of leaves of the nonirradiated control plants was expanded and the second pair was partially expanded. The first pair of leaves of the 20-hour irradiated plants was only very slightly mottled at this time and was free of marginal serration or any other abnormal formative effects. The second pair and all subsequent pairs of leaves developed at the same rate as those of the control plants and were normal in every respect. The slight mottling in the first pair of leaves became even less noticeable as time progressed.

Noticeable damage at the 15th day was more severe in the 25-hour plants; the size of the first pair of leaves was comparable to that of the 20-hour plants but mottling was pronounced; they were lighter in color and their margins were serrated. A few days later light streaks and blotches appeared in their leaves, and the plants were slightly stunted in height compared to the nonirradiated control plants as well as the 20-hour irradiated plants. The second pair of leaves of the 25-hour plants was only mildly mottled and showed irradiation effects very similar to those shown by the first pair of leaves of the 20-hour plants. The third pair and all subsequent leaves of the 25-hour plants were free of all irradiation effects.

The 42-hour plants were most severely damaged of all lots indicating high radiosensitivity at this stage. The first pair of leaves at the 15th day was one-half the size of those of the 25-hour plants; the leaves were extremely mottled, had serrated margins, and were misshapen. The plants were also stunted in height more so than plants of the preceding lot. When the second and third pairs of leaves developed, they were also found to be reduced in size, bleached, distorted and serrated. All subsequent leaves as late as the 29th day were similarly affected and on the 57th day, when all plants were discarded, the newest formed leaves were still much reduced in size; some were strap-like and others were distorted and bleached, although some were more or less normal in appearance.

The 49-hour irradiated plants developed marked symptoms but appeared considerably less damaged than the 42-hour plants at the 15th to 18th days. Their first pairs of leaves appeared quite similar to the first pairs of the 25-hour plants in size, shape, and color, and the plant heights of the two were also similar. By the 22nd day, however, it was apparent that the 49-hour plants were more severely affected than the 25-hour plants but considerably less so than the 42-hour plants; their first, second, and third pairs of leaves were bleached and misshapen. While the 25-hour plants produced normal leaves starting with

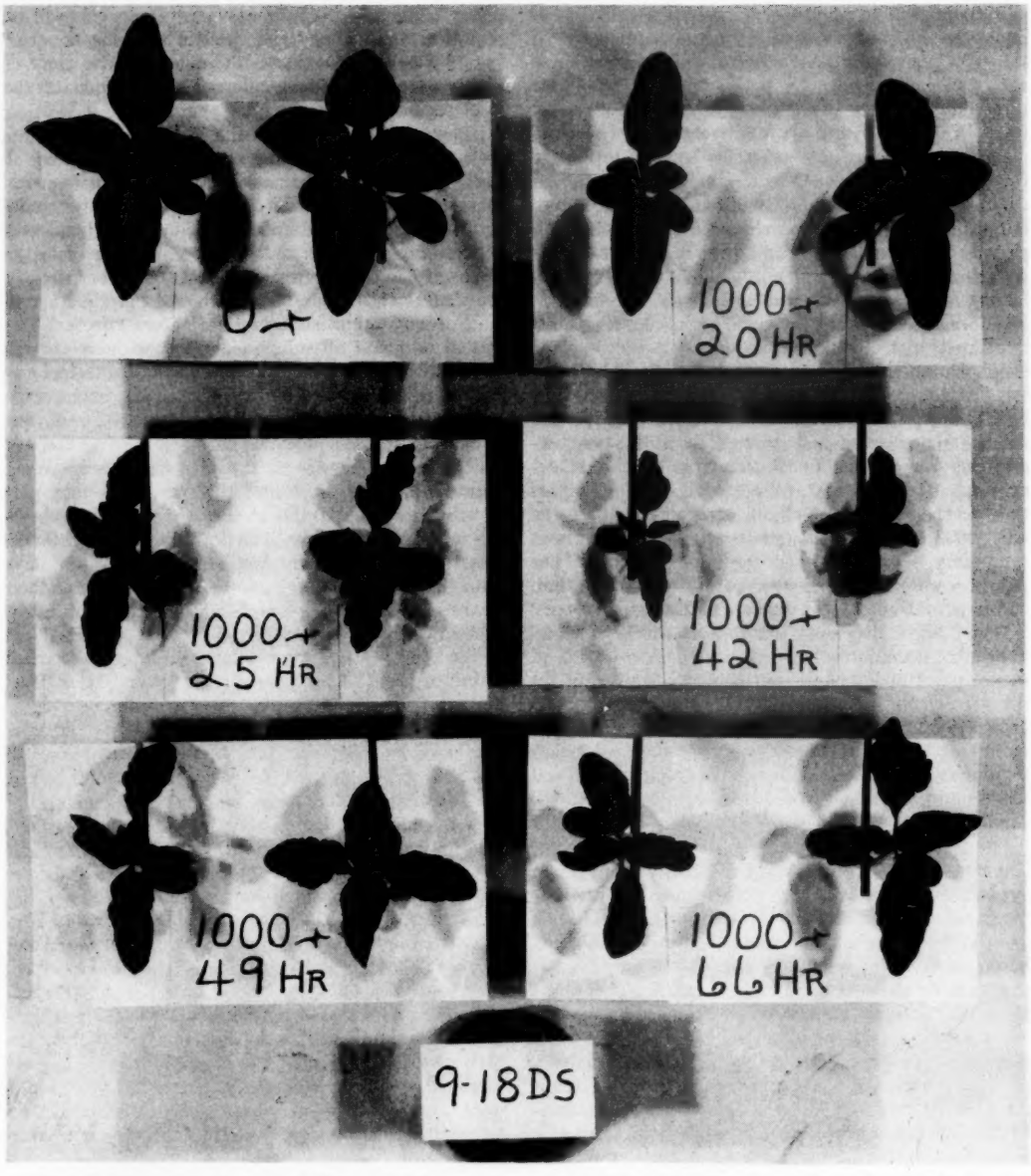

FIG. 5. Top view of plants 18 days after time seed were started to soak. Top row, left, nonirradiated control; all others x-irradiated with 1000 r at (left to right and top to bottom) 20, 25, 42, 49, and 66 hrs after start of soaking of seed.

the third pair, these 49-hour plants continued to produce affected leaves; at the 29th day considerable distortion and streaking was present in the newest formed leaves and even at the 57th day the newest leaves of some plants continued to show radiation effects.

The radiation-induced effects of the 66-, 73-, 90-, and 97-hour plants were essentially similar and indistinguishable. The general severity of the effects in these later groups was less than that in the 49-hour plants and very much less than that in the 42-hour ones, but greater than that in the 25-hour groups and very much greater than that of the 20-hour lot. The first pair of leaves showed moderately severe mottling, bleaching, and marginal serration. These same symptoms appeared in the second and third pairs of leaves

but to a slightly lesser degree, although the radiation-induced leaf symptoms persisted. The most recently formed leaves at the 29th day were of about normal size but many were distorted, bleached, and buckled; at the 57th day the newest formed leaves in some of the plants still showed irradiation effects.

This irradiation series indicates that there is a marked and measurable change in radiosensitivity during the early stages of germination and seedling development. During the very early part of germination the cells stretch and swell on taking up water, and then cell division becomes a predominant phase of cellular activity; this phase (cell division) is marked by relative radioresistance. Cell division is followed by cellular maturation and differentiation (although cell division continues); this later period is characterized by marked radiosensitivity. This rise in radiosensitivity (which can be lowered by withholding boron during the later seedling stage) suggests a boron requirement for some process or processes concerned with cellular maturation or differentiation. This conclusion, of course, cannot be considered an unequivocal one, since there may be other ways in which boron can alter radiosensitivity.

Relationship between Cellular Activity and Sugar Translocation: It has been proposed (1, 2) that a major function of boron is its direct influence on sugar translocation, in that a sugar-borate complex diffuses more readily through membranes. Recent experiments conducted at this laboratory (9) have indicated a relationship between the complexing property of the borate ion with polyhydroxy compounds and the function of boron in plant growth, but they did not support the hypothesis that this relationship is directly involved with sugar translocation. These present experiments emphasize the important relationship between boron and cellular activity. It appears that the apparent enhancement of sugar translocation by boron is related to cellular activity rather than directly to the formation of a boron-sugar complex. Materials, including sugar, move from leaves to such metabolically active regions as growing tips because of a gradient. The active regions utilize sugars faster both because of a high respiration rate and a high growth rate accompanied by rapid utilization of carbohydrates in syntheses, thus depleting sugar in these regions. In the meristem and terminal region of a boron-deficient plant, metabolism is at a lower rate and translocation of sugar from leaves to this region is reduced; the addition of boron would again raise the metabolic rate and increase movement of sugars into the area. The high variability usually seen in the results of sugar translocation experiments following the addition of boron is perhaps related to the variability in the amount of boron already present in the plant. This in turn would determine whether additions of boron to the plant would have any effect in increasing the activity of the meristem.

The indirect relationship of boron to sugar translocation was demonstrated in an experiment designed to determine the rate of translocation of C^{14} sugar applied to leaves in relation to the metabolic activity of the apical meristems and terminal bud regions. Sunflower plants were grown in + B nutrient solutions for 9 days. At the 9-day growth period 18 uniform plants were selected and divided into 3 groups of 6 plants per group. Group I: intact plants were transferred to fresh + B nutrient solutions; Group II: intact plants were transferred to – B solutions; Group III: plants were transferred to + B solutions and, 24 hours prior to application of C^{14} sugar, their terminal buds were excised. The last treatment was included to determine whether sugar translocation in plants adequately supplied with boron is retarded by the removal of the active tip region. The plants transferred to – B solutions (Group II) were first grown on + B solutions so that they would make a considerable amount of growth before a partial boron deficiency was induced. The sugar was applied on the fourth day in the – B solutions. At this time a total of 100 μl of 5 % dextrose solution labeled at 1 mc/gm (containing a total of 5 mg sugar and 5 μc of radioactivity) were administered to each plant of all three lots by applying 50 μl inside vaseline rings on each leaf of the first pair. Twenty-seven hours later the plants were harvested. The first pair of leaves, which received the sugar applications, and the cotyledons, were excised and discarded. The plant axes were severed at the second node (where the first pair of leaves was attached) and at the junction of the hypocotyl and roots, and each plant was divided into three parts: 1) tops, which included all leaves (excluding the first discarded pair) and stem from the second node up; 2) lower axis, which included the first internode and hypocotyl portion to root region; and 3) roots. The tissues were separately oven-dried, ground, and counted in the manner previously described (9).

The assay values of the various tissues indicate (table I) that sugar translocation into the top portions was significantly reduced in + B plants with excised growing tips; it was only 57 % of that translocated to the top portions of intact plants. Translocation into the top portions of the – B plants was 75 % of that of control plants, but the reduction was not

Table I

Translocation of C^{14} Dextrose Applied to Lower Leaves of Sunflower Plants (Relative Count Rate ± S.E. of Mean) *

Lot	Tops	Lower axis	Roots
1. + B, intact	100 ± 14	100 ± 14	100 ± 14
2. – B, intact	75 ± 22 (0)	102 ± 38 (0)	84 ± 20 (0)
3. + B, tips excised	57 ± 11 (S)	109 ± 16 (0)	121 ± 25 (0)

* Relative count rate of 100 is equivalent to 551, 950, and 954 μgm dextrose per gram dry tissue in case of tops, lower axis and roots respectively. Figures in parentheses refer to significance of count rate differences when compared to No. 1: 0, not significant; S, significant at 5 % level.

statistically significant in this case, again primarily because of variation between individual plants. The quantities of C^{14} in the lower axes did not differ greatly in the three lots. The roots of the – B plants contained smaller amounts of translocated sugar than those of intact + B plants, but differences were not significant; as to be expected, the levels of sugar in the roots of the + B plants with excised tips were not reduced but in fact were somewhat elevated.

Discussion

Withholding boron prior to irradiation resulted in a retardation of cellular activity with an accompanying reduction in radiation damage. The phase of cellular activity which is normally radiosensitive and which can thus be rendered relatively radioresistant, then, is boron-dependent. If the identification of this normally radiosensitive phase as the phase of cellular differentiation rather than cell division is correct, it would indicate that boron is required for some process or processes concerned with cellular maturation or differentiation rather than for cell division. Although dividing cells have generally been considered to be most radiosensitive (10, 12), Quastler et al (3) have also noticed a developmental rise in radiosensitivity in successive stages of germinating mung beans and have found differentiating cells to be much more radiosensitive than dividing cells (4, 5). Of additional interest is a similar finding in the animal kingdom. In the grasshopper embryo Tahmisian et al found both tissue differentiation (13) and cellular differentiation (Tahmisian, personal communication, 1957) to be more radiosensitive than cell division.

As dividing and differentiating cells are generally in close proximity to each other in actively growing tissues, it is often difficult to recognize clearly by casual observation the cellular stage which is the first to be involved in the early cellular damage resulting from boron deficiency. The inference is generally made that boron is a growth requirement and needed for cell division, since necrosis in apical and root meristems is a typical boron deficiency symptom. Reed (6), however, has shown that in the apical portions of boron-deficient sunflower and celery plants, the sub-apical cells were first affected while the cells of the primary meristem were normal; the terminal cells became necrotic only after those below had failed. These observations then also would indicate that cell division can proceed in the absence of boron although the completion of the growth cycle (cellular maturation) is prevented; the failure of the underlying tissues, then, results in death of the entire meristem. The writer (9) has previously suggested that the complexing property of the borate ion appears to be related to the formation of some structural unit, which is in agreement with similar earlier suggestions made by Schmucker (7) and also by Torssell (14). Schmucker proposed that the boron-complexes may be directly related to the formation of membranes. Torssell, after finding that a number of arylboric acids possessed growth-promoting effects on wheat roots by their promotion of cell elongation, suggested that the complexing property of boric acid, as well as that of these acids, may be involved in regulation of the formation of the cell wall. These concepts are all consistent with the present radiation response data.

The boron requirement for cellular differentiation may even be specific in that differentiation is boron-dependent only in certain cell types. This possibility merits some consideration, since such specificity might be the basis for the essentiality or non-essentiality of boron among the various plant groups. The vascular plants all appear to need boron; vascular tissue is generally affected by boron deficiency and mature plant tissues including lignified tissues are high in boron content, although such mature tissues as fully expanded leaves are little affected if boron is subsequently withheld after these organs have been fully developed. Boron is required for the formation of tissues but does not appear to be essential for the maintenance of tissues already matured (see fig 6), and little or no reutilization of boron occurs. Fungi do not appear to require boron, but unfortunately little information is available concerning the lower forms as a whole. Determination of boron requirements in specific types of plant cells by means of the tissue culture technique might prove useful in an examination of possible specificity of boron dependence in the differentiation of various cell types. The characteristics of the plant cell wall as contrasted with the animal cell wall might well be the basis of the essentiality of boron in higher plants and its non-essentiality in animals. If boron were needed for some other basic physiological function in the cell, it would be reasonable to expect its requirement to be more widespread among living organisms. Also, if boron were of importance in facilitating diffusion of sugars through membranes, it would be expected to have equal importance in animal and plant cells.

The radiation responses as affected by time of irradiation of the germinating seedling and the alleviation of radiation damage by withholding boron permit a reasonable conclusion as to a probable role of boron in the plant cell. There are, however, two responses that cannot be adequately explained. It is not clear why, after radiosensitivity in the germinating seedling rises during the maturation stage to a high point at the 42-hour irradiation period, it then declines again slightly. Of course, it declines only slightly and not to the early low level (the 20- and 25-hour periods). The second response for which an adequate explanation would be even more desirable involves the radiation responses in relation to boron. It is postulated that withholding boron temporarily prior to irradiation with 1000 r reduces the rate of cellular maturation and renders these cells radioresistant. Such plants develop symptoms whch do not persist beyond the 6th to 7th pair of leaves. The question then arises as to how injury to some process or processes involving cellular differentiation and maturation persists normally. A persistent effect, one

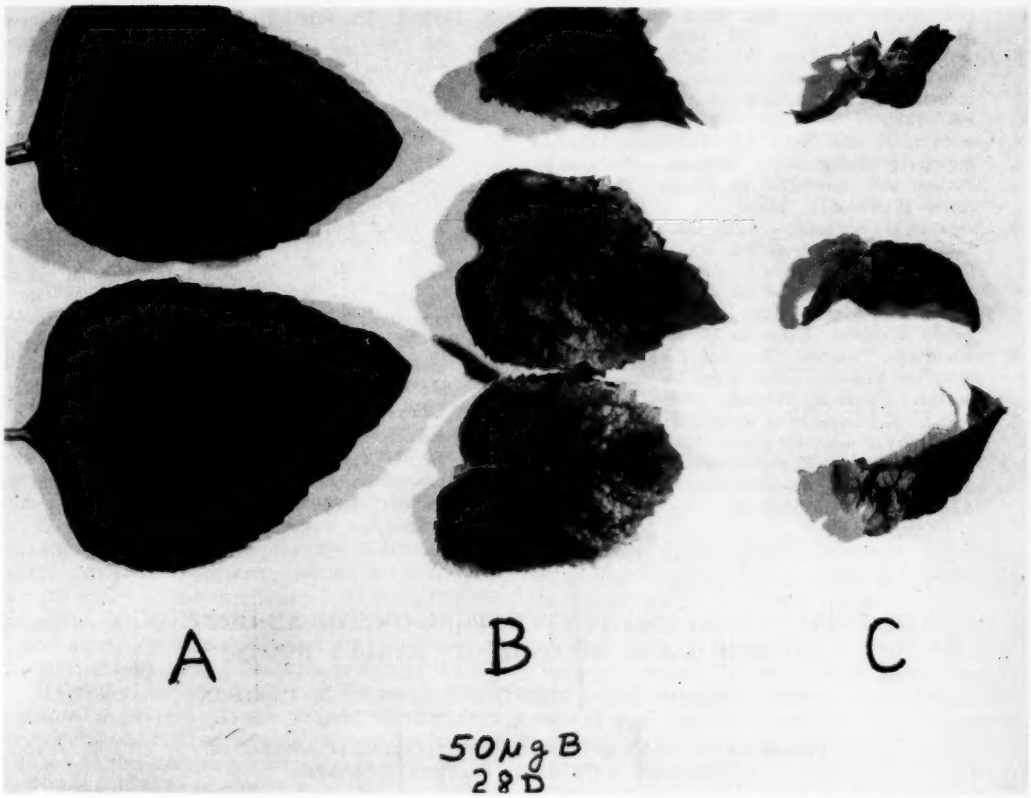

FIG. 6. Plant parts at the 28th day of growth in nutrient solutions containing a total of 50 µgm boron. A, old leaves taken from 3rd node which matured before available B was depleted, showing no abnormal symptoms; B, leaves from the 4th and 5th nodes which developed during time when available B was sub-optimal, showing severe symptoms; C, tips and youngest leaves, dead. Boron deficiency symptoms become apparent at about the 18th to 19th day when the supply of B is limited to 50 µgm per plant.

which is passed to succeeding cells, it seems, would be generally expected to involve nuclear material although plastids or other cytoplasmic entities may be involved. A clear understanding of this phenomenon will perhaps become attainable as a better general understanding of radiobiological effects becomes manifest.

SUMMARY

Seven-day-old Mammoth Russian sunflower seedlings were grown in nutrient solutions with or without boron, for 2 to 5 days, after which they were x-irradiated and again supplied with boron. Withholding boron for short intervals prior to x-irradiation with doses that normally produce pronounced radiation symptoms, had a marked alleviating effect on the development of radiation injury. The absence of boron resulted in retardation of some phase of cellular activity and rendered the plants more radioresistant. This normally radiosensitive phase of cellular activity which may be rendered relatively radioresistant by withholding boron is, then, boron-dependent.

An attempt was made to identify this growth phase by irradiating a series of germinating seedlings at different growth stages. It appeared to be the phase of cellular maturation rather than cell division, suggesting that boron is required for some process or processes concerned with cellular maturation or differentiation.

The apparent effect of boron on sugar translocation appears to be related to the effect of boron on cellular activity rather than to a direct enhancement of diffusion through membranes through formation of a sugar-borate complex.

I wish to thank Dr. Henry Quastler for several interesting and helpful discussions we had pertaining to this work.

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THE EFFECT OF pH AND TEMPERATURE ON THE ABSORPTION OF POTASSIUM AND BROMIDE BY BARLEY ROOTS[1,2]

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It has long been recognized that the process of ion absorption has optimum ranges of pH and temperature, i.e., the physiological pH and temperature range. In general, there has been a tendency to assume that the factors of pH and temperature are not important provided they fall within the physiological range. However, marked changes in the pH of the culture media can result due to unequal absorption of cations and anions. This suggests that H^+ and OH^- are involved in the absorption process and accordingly they have been given a key role in a generalized expression for the absorption of cations and anions (9). This expression involves a metabolically produced ion carrier whose rate of production is dependent upon those factors which effect metabolism such as temperature, O_2, substrate, etc.

For these reasons and because previous experience has led us to believe that pH and temperature, even within the so-called physiological range may exert a much higher degree of influence than generally suspected, absorption experiments have been carried out with KBr in which pH and temperature were rigidly controlled.

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Materials and Methods

Excised root material from Tennessee Winter barley 1954 crop, prepared as previously described (9) was used for these experiments. In all experiments, 7 grams of root material in 7 liters of solution was used. An absorption period of 3 hours was adopted throughout. During the course of the experiments, the solution was aerated with CO_2-free air. The concentration of KBr in all culture solutions was nominally 0.005 M. During the absorption period, the pH of the solutions was frequently checked and maintained by the addition of KOH or HBr. The maximum deviation from a given pH throughout an experiment was never more than 0.1 pH unit. The small changes in concentration caused by the addition of KOH or HBr were negligible and were found, by supplementary experiments, to have no detectable effect on the rate of absorption. The absorption vessels were immersed in a water bath in which the temperature was controlled within 0.1° C.

At the conclusion of an experiment, the roots were separated from the culture media by means of a nylon mesh filter and washed for 10 seconds in running distilled water. After this period of washing, the roots were found to possess an apparent free space of 18 % as determined by leaching experiments. Potassium was determined by a flame photometric proce-

dure and bromide by oxidation to bromate (12). All data are expressed in milliequivalents per kilogram fresh weight of roots and have been corrected for apparent free space.

Results

The absorption of K^+ and Br^- as functions of pH were determined at fourteen different temperatures from 0° C to 50° C. The essential character of the interaction of temperature and pH on K^+ absorption is shown by the five curves of figure 1. In all cases K^+ was lost from the roots at the lower pH values. At any given pH, the loss was markedly modified by temperature. In general, the lower the temperature, the lower the pH at which K^+ was neither lost nor gained by the roots. This is in essential agreement with previous results (9). Of particular interest is the fact that at most temperatures, absorption is not reduced even at pH values of 10. Only when the temperature exceeded 37.5° C was the absorption observed to drop markedly at the high pH values.

The picture for the temperature and pH relationships for Br^- absorption is presented in figure 2. As in the case of K^+ absorption, less Br^- is taken up at low pH values at all temperatures and, at a given pH, the uptake of Br^- is modified by temperature. The major difference between K^+ and Br^- absorption is the greater sensitivity of the latter to high pH values.

Absorption curves for K^+ and Br^- at 35° C are presented in figure 3. At this temperature, the maximum uptake of the two ions are very similar in magnitude and the absorption curves can be most readily compared. It may be clearly seen that Br^- uptake falls off rapidly at pH values above 5 whereas K^+ uptake is but little affected. Below pH 5, the absorption of both ions falls off rapidly but that of K^+ shows a sharper decrease; that is, K^+ absorption appears to

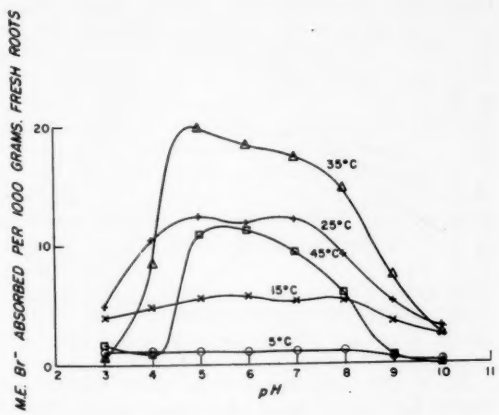

Fig. 2. The effect of pH on the absorption of Br^- from solutions of 0.005 *N* KBr at different temperatures. Three-hr absorption period.

be somewhat more sensitive to lowered pH. Essentially similar conclusions can be drawn by comparing the absorptions at other temperatures.

In figures 4 and 5, the effect of temperature on the absorption of K^+ and Br^- at four selected pH values is presented. In constructing these curves, the data for the fourteen different temperatures were used. It is evident from figure 4 that the temperature optimum, at least for a 3-hour absorption period, of K^+ uptake shifts with increasing pH. At pH 4, the optimum is about 22° C, at pH 6, the optimum is 30° to 35° C. Other data, not presented in these curves, indicate that the optimum tends to move back to lower temperatures as the pH increases above 6. The curves in figure 4 show a considerable amount of ir-

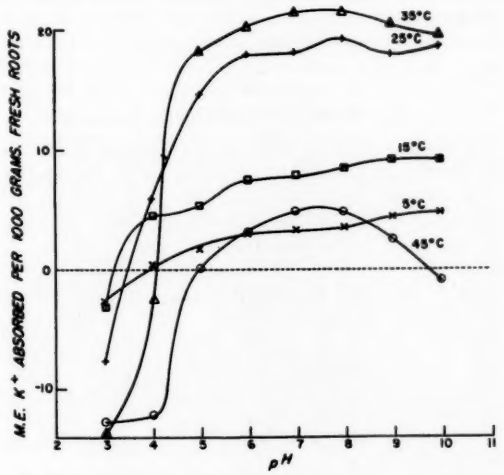

Fig. 1. The effect of pH on the absorption of K^+ from solutions of 0.005 *N* KBr at different temperatures. Three-hr absorption period.

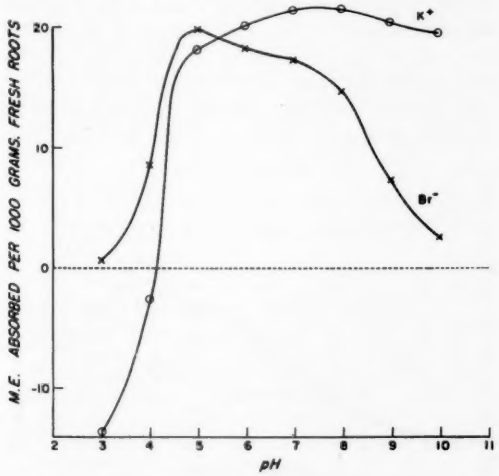

Fig. 3. The effect of pH on the absorption of K^+ and Br^- from solutions of 0.005 *N* KBr at 35° C. Three-hr absorption period.

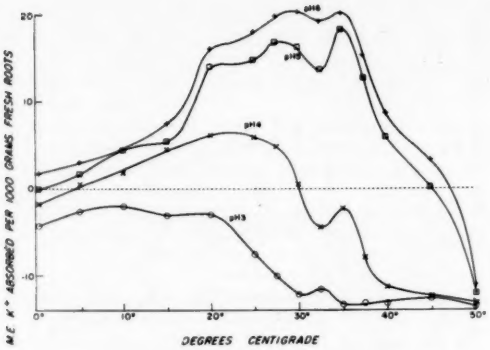

FIG. 4. The absorption of K^+ from solutions of 0.005 *N* KBr as a function of temperature at different pH values. Three-hr absorption period.

regularities which are most probably real since a given irregularity may be repeatedly obtained.

The Br^- data, presented in figure 5, also indicate a shift of optimum temperature with pH, the optimum temperatures being a little higher than in case of K^+ uptake. At pH 3, the optimum is about 23° C and at pH 5, the optimum is about 37° C. Again at pH values above 5, the optimum shifts back to lower temperatures.

DISCUSSION

Before discussing the data, it is perhaps pertinent to consider the question of "apparent free space" as it applies to absorption data. It has been found that a fraction of the ions taken up may be readily removed by subsequent treatment with distilled water. Recently a number of investigators have reported that roots have an apparent free space for solutes and a fraction of the solutes entering a root does so by non-metabolic means (2, 7). The amounts of K^+ and Br^- observed in this research to be easily removed by water treatment agree approximately with values reported by the above investigators. We have corrected the absorption data for these loosely held ions without, for the time being, committing ourselves as to the interpretation of this fraction of the uptake. However, it should be emphasized that these corrections are quite minor and have a negligible effect on the character of the absorption curves except under conditions of relatively low uptake. In general, when the concentration of the absorbed ions becomes large relative to that of the culture medium, the apparent free space correction is of little importance in so far as the absorption data are concerned. It is only when the concentration of the absorbed ions in the tissues is of the same magnitude as or is less than that of the external solution that apparent free space assumes important proportions.

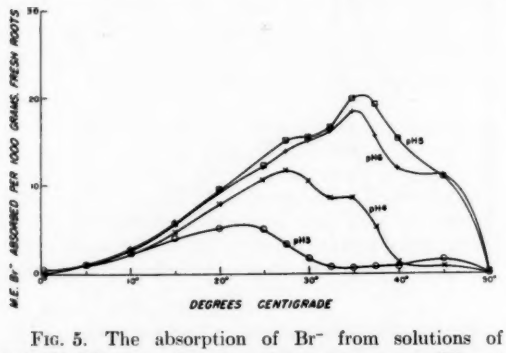

FIG. 5. The absorption of Br^- from solutions of 0.005 *N* KBr as a function of temperature at different pH values. Three-hr absorption period.

Attempts have been made to utilize Q_{10} values of absorption as an aid in establishing the mechanism of the absorption process (10, 11). An examination of the data reported here reveal a marked dependence of Q_{10} on pH as well as on temperature range. Moreover, it is likely that this dependence will vary with different cations and anions. For these reasons, the comparison of Q_{10} values for an anion and a cation at a given pH and temperature range probably is of little general significance.

Of particular interest is the relationship between the amounts of cation and anion uptake as a function of pH and temperature. By the appropriate selection of pH and temperature, it is possible to have excess K^+ absorption, excess Br^- absorption or equal absorption of K^+ and Br^-. These separate conditions would result in quite different chemical changes in the root (8). Thus it appears that the pH and temperature of the external solution have a major influence on the biochemistry of the root.

The data presented in the curves have interesting implications concerning the mechanism of absorption. In a previous publication (9), it was suggested that absorption could best be described by the following expressions.

$$M^+ + HR = MR + H^+ \text{ for cations}$$

and

$$A^- + R'OH = R'A + OH^- \text{ for anions}$$

Our present view is that these equations should be considered as generalized formulations of the absorption process. It is quite possible that they do not represent specific chemical reactions. More than one reaction and more than one compound may be involved for each ion. The value of the above expressions is that they conveniently summarize the overall features of mineral absorption. That is, they indicate the necessity of a carrier produced by the plant and the requirement for electrical balance both within the plant and the culture media. Furthermore, they emphasize the importance of H^+ and OH^- and they allow for a degree of independence of cation and anion absorption.

The data for the absorption of K^+ and Br^- are consistent with this scheme. Br^- absorption is markedly reduced as OH^- concentration increases whereas K^+ absorption is little affected. At low pH values, the

absorptions of both K^+ and Br^- are markedly reduced. However at the very low pH values, the situation is complicated by tissue injury (9). In spite of this, K^+ absorption is more affected by increased H^+ concentration than Br^- absorption. Two explanations of this present themselves; 1) the cation absorbing system is more subject to H^+ injury than the anion absorbing system, or 2) both systems are equally subject to H^+ injury but the more rapid decrease of K^+ absorption at the lower pH values represents a competing effect of H^+ for K^+. If the latter is true, then the losses of K^+ which occur under conditions where Br^- is still being absorbed, i.e., figure 3, may represent, in part, a reversal of absorption although not necessarily through the same pathway that uptake occurs (1).

The concept of reversibility of accumulation has recently been challenged by Epstein on the basis of short term translocation experiments with radioactive isotopes (3). However, there appears to be substantial evidence that the accumulation process is reversible. That is, it has been well established that roots may be depleted of previously accumulated ions by translocation to the shoots. Also it has been commonly observed that older leaves are depleted of certain accumulated ions by new growth. A good example of such evidence is that of Woolley et al (13) showing that Cl^- which has been accumulated by the root is subsequently lost from the root by translocation. Since this occurred at a time when the culture media was essentially free from Cl^-, it would appear to be conclusive evidence that root cells may lose previously accumulated ions in the normal course of growth.

The results of this research show that such factors as temperature and pH have a profound influence on the absorption process. Furthermore past experiments have shown a marked interaction between the ion under study and other cations and anions which may be present (5). This interaction may take the form of a stimulation or depression of absorption which is pH dependent. All of these influences apply to roots grown from a given sample of seed.

It has been found that each sample of seed gives roots having a somewhat different absorption behavior. Even using the same variety will not insure consistent behavior. For example, roots obtained from a sample of Sacramento barley seed 1951 crop showed a considerably greater reduction of K^+ uptake at low pH values than those obtained from the 1948 crop of the same strain grown at the same place. Such factors as pH, temperature, concentration, etc. affect the absorption process in different varieties or even in different samples of the same variety in only roughly a similar manner. Large quantitative as well as qualitative differences are frequently encountered.

Even when a series of experiments is conducted with a selected batch of seed, it has been found necessary to consider the factor of age of the seeds, particularly when an extended period of time is involved. That is, the absorption characteristics of roots grown from a sample of seed will begin changing after a period of seed storage and moreover the change may manifest itself at certain temperatures and pH values and not at others. For example, a sample of Atlas 46 barley, after storage for 2 years, began to display a markedly enhanced uptake of Br^- at pH 5 and at 35.5° C, although at 25° C, its absorption behavior did not differ appreciably from that observed previously. It has therefore been found necessary to check the absorption characteristics at different temperatures and pH values, from time to time, to verify constancy of behavior during the course of a series of experiments.

Thus any satisfactory mechanism of absorption must allow for the interplay of all the various factors mentioned above. It must not be limited to a narrow range of environmental conditions nor to any specific sample of material. As an experimental procedure, it would appear unwise to explain the kinetics of absorption solely or largely by means of mathematical analyses of absorption curves obtained under a given set of conditions as has been attempted recently (4, 6). That is, we must conclude that it is highly unlikely that a mechanistic and quantitative picture of absorption based on apparent similarities to known kinetic models and dependent upon restricted experimental data will be generally applicable.

Summary

The influence of pH and temperature of the culture media on the absorption of K^+ and Br^- by excised barley roots was studied. These two factors were shown to have marked and differing effects on the uptake of the two ions. The significance of apparent free space as a correction factor was briefly discussed. When material possessing a high absorption capacity is used, the correction due to apparent free space appears to be insignificant in most cases. On the basis of the experimental data and other considerations, it is concluded that the accumulation process is reversible.

The significance of Q_{10} values and the applicability of kinetic absorption models are discussed in terms of the experimental results.

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METABOLIC PROCESSES IN CYTOPLASMIC PARTICLES OF THE AVOCADO FRUIT. IV. RIPENING AND THE SUPERNATANT FRACTION[1]

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The activities of the cytoplasmic particles reported in the first three papers of this series (1, 4, 5) were obtained mostly from firm or pre-climacteric fruit. Little or no attention was paid to the mitochondrial reactions of ripe fruit and of fruit in the course of ripening. In the avocado, a well described pattern of respiration for the intact fruit is associated with the ripening process (2). This pattern is essentially the same as that reported for other fruits and is referred to as the "climacteric" (10). Under appropriate temperature and oxygen tension, a rapid rise in respiratory activity takes place within a short time after harvesting. This so-called "climacteric rise" reaches a peak and is followed by a decline referred to as the post-climacteric phase. It is during this final stage that senescence sets in, resulting in breakdown and death. Fruit physiologists have been concerned with the onset of the climacteric rise since it might be considered as the "beginning of the end," the prelude to senescence and death. Recent hypotheses concerning the onset of the rise revolve around the phenomenon of the coupling of phosphorylation to respiration (13) or the relationship between the levels of ADP and ATP (14). It is the purpose of this paper to examine the biochemical reactions of avocado particles prepared from fruit at different stages of the climacteric.

Materials and Methods

The Fuerte and Anaheim varieties of the avocado, *Persea americana* Mill, were used, but no substantial qualitative or quantitative differences between these varieties were observed. Respiration of the whole fruit was determined by methods described by Biale and Shepherd (3), with the exception that oxygen consumption was measured with the Beckman Oxygen Analyzer (19). The preparative procedure and the determination of oxidative and phosphorylative activities for hard fruit were described previously (4). In brief, the method consisted of blending 150 grams of peeled and grated avocado tissue with 300 ml of 0.5 M sucrose. The homogenate was then strained through cheesecloth and centrifuged at 500 × g for 5 minutes to separate out large fragments, unbroken cells, etc. The resultant supernatant solution was then centrifuged at 17,000 × g for 15 minutes. After this high-speed centrifugation, the supernatant solution was saved for further study and the cytoplasmic particles contained in the pellet were washed by resuspension in 0.5 M sucrose and recentrifuged at 17,000 × g. In the case of soft fruit, the tissue was prepared with a coarse grater. The speed of blending was adjusted so as to prevent the formation of a suction cone in the homogenate.

A major portion of this study was concerned with the effects of the deproteinized supernatant fraction on ripe fruit mitochondria. For this purpose, the supernatant solution resulting from the first high speed centrifugation was placed in conical centrifuge tubes and the proteins were denatured by immersing in boiling water for five minutes. Coagulated proteins were removed by centrifugation at 1000 × g for five minutes.

The following abbreviations are used in this paper: DNP, 2,4-dinitrophenol; DS, deproteinized supernatant fraction; KG, α-ketoglutarate; AMP, adenine monophosphate; ADP, adenine di-phosphate; CoA,

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coenzyme A; LTPP, lipothiamine pyrophosphate (kindly supplied by Dr. D. Appleman).

Results

Oxidative Phosphorylation During Ripening: It was established in previous studies (4, 13) that the particulate fraction of the avocado is capable of converting inorganic phosphate into organic form as a result of the oxidation of α-ketoglutarate. While some evidence was furnished that oxidative phosphorylation took place at two stages of the climacteric, the data were limited to one keto-acid. Moreover, coordination of mitochondrial studies with respiratory measurements on the whole fruit was lacking. The magnitudes of both oxygen and phosphorus uptake with the preparations made by grinding in a mortar (13) were much lower than those obtained by our current blender technique. We decided, therefore, to use the present preparative procedure on fruit with a well known respiratory history and to follow the rates of oxidation and phosphorylation in the absence and presence of dinitrophenol. The experimental results presented in table I are based on averages of two or more tests for each stage of ripeness and for each acid. The 40-minute period was chosen since both phosphate and oxygen uptake were constant during this time (4).

The mitochondrial oxidations and phosphorylations in reaction mixtures without DNP (control) will be examined first. The oxidative activities of the succino-oxidase system were stable throughout the climacteric cycle. In the case of the other three acids there was a sharp drop in the rates of oxidation at the peak (maximum) as compared with activities of material from the preclimacteric minimum. On the other hand, the rates of incorporation of inorganic phosphate were essentially not lowered during the climacteric rise. Consequently the P/O values increased with ripening.

The trends were in some respects different for the preparations treated with 10^{-5} M DNP. This reagent lowered more markedly the phosphate uptake by cytoplasmic particles from unripe (initial and minimum) than from ripe fruit. This observation was not as manifest with α-ketoglutarate as with the other three acids of the Krebs cycle. The uncoupling action of DNP was most striking in the mitochondria from hard fruit. In no case did DNP bring about an increase in oxidative activity. In all cases, with the exception of succinate, the rates of oxidation were distinctly lower at the maximum than at the other two stages of ripening. Apparently the trend in oxygen uptake by the mitochondria did not correspond with the course of respiration exhibited by the intact fruit.

Since the DNP effects with pyruvate were striking, another experiment was undertaken in which the P/O ratios were determined in the intermediate stages as well as at the climacteric minimum and maximum. The results of this experiment are depicted in figure 1. Curve A in this figure represents the respiration of the intact fruit as measured by the oxygen analyzer. Each point in curves B and C was obtained from a fruit the respiration of which is given by a corresponding point in curve A. The findings in this experiment extended the results of table I and indicated the effectiveness of DNP in altering P/O ratios in the preclimacteric stage and immediately after the onset of the rise. It should be stressed again that DNP had no effect at and following the peak. This behavior of DNP suggested that marked biochemical changes occurred and justified further investigation.

Oxidation of Succinate and α-Ketoglutarate in Relation to Stage of Ripeness: Succinate and α-ketoglutarate were chosen to test metabolic activity during ripening because previous evidence (4) indicated wide differences in the stability of these two

Table I

Dinitrophenol Effect on Oxidative Phosphorylation by the Particulate Suspension of Avocado Fruit in Relation to Stage of Ripeness

Substrate	Stage of ripeness	Control μatoms/40 min		P/O	Dinitrophenol (10^{-5} M) μatoms/40 min		P/O
		P	O		P	O	
α-Ketoglutarate	Initial*	15.4	14.9	1.04	6.7	15.4	0.44
	Preclim. min.	12.8	13.9	0.92	3.1	11.8	0.26
	Clim. max.	11.6	8.0	1.45	4.6	5.1	0.96
Pyruvate	Initial*	5.0	5.2	0.96	0	4.5	0
	Minimum	11.7	15.7	0.75	3.1	16.4	0.19
	Maximum	9.0	6.0	1.50	4.6	5.5	0.83
Succinate	Initial*	7.4	15.4	0.48	1.5	16.8	0.09
	Minimum	10.3	18.0	0.58	4.3	16.6	0.26
	Maximum	13.1	15.7	0.84	10.5	16.2	0.65
Malate	Initial*	10.4	14.7	0.72	3.1	15.1	0.21
	Minimum	11.5	16.5	0.70	1.5	14.2	0.11
	Maximum	10.6	7.7	1.38	8.9	7.0	1.27

Reaction mixture: Substrate, 0.02 M; phosphate, 0.01 M, pH 7.1; AMP, 0.001 M; glucose, 0.01 M; magnesium sulfate, 0.006 M; sucrose, 0.5 M; enzyme, 0.5 ml (approx. 1 mg N); total volume 3.0 ml; gas phase, air; temp 20° C.
* Refers to fruit fresh from the tree.

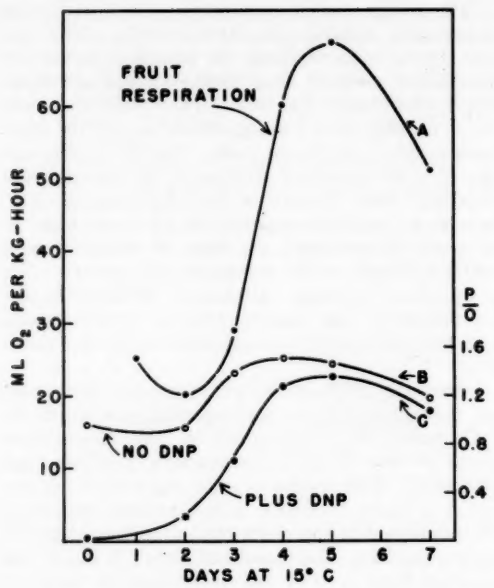

FIG. 1. Effect of 10^{-5} M dinitrophenol on oxidative phosphorylation by cytoplasmic particles of the avocado fruit in relation to the climacteric. A. Oxygen uptake by the intact fruit. B, C. P/O ratios of particles from fruit of curve A on corresponding days. Reaction mixture same as in table I; substrate, pyruvate.

systems. Two sets of experiments were conducted. In the first set the fruit was kept at 0° C for different periods of time and homogenized immediately upon removal from the low temperature. In the second set homogenization was carried out only following the exposure to the ripening temperature of 20° C. It is evident from table II that succinate oxidation was virtually unaffected by either treatment. On the other hand, the rates of oxidation of α-ketoglutarate were materially lowered by chilling fruit at 0° C. The expected Q_{O_2} (N) value for this substrate in the case of non-chilled fruit is at least 150, as judged from table I and from results reported previously

TABLE II

MITOCHONDRIAL ACTIVITY IN RELATION TO FRUIT STORAGE AT 0° C AND AT 20° C

FRUIT TREATMENT	Q_{O_2} (N)	
	SUCCINATE	α-KETOGLUTARATE
8 Days at 0° C	266	68
18 " " "	430	94
30 " " "	338	93
Chilled at 0° C and placed		
3 Days at 20° C	387	38
8 " " "	415	20
15 " " "	250	6

Reaction mixture: Same as in table I plus 3 micromoles cytochrome c.

TABLE III

EFFECT OF THE SUPERNATANT FRACTION ON α-KETOGLUTARATE OXIDATION

STAGE OF RIPENESS	MG N/ VESSEL	CONDITION	Q_{O_2} (N)	% INCREASE
Preclimacteric	0.58	Control	427	...
		DS **	410	−4
On rise	0.52	Control	105	...
		Fresh supn't.*	189	80
		DS **	248	138

Reaction mixture in control: same as in table I. Temp. 30° C.

* Five tenths ml of supernatant fraction obtained from high speed centrifugation (see text).

** DS, deproteinized supernatant fraction.

(4). The striking feature of table II is the progressive inactivation of the α-ketoglutarate oxidase with time of exposure to 20° C. These results along with those in table I suggested the need to investigate further the decline in α-ketoglutarate oxidation in relation to ripening.

ENHANCEMENT OF OXIDATION BY A SUPERNATANT FRACTION: Millerd, Bonner, and Biale (13) supplied evidence that a supernatant factor from ripe fruit increased the oxidation of α-ketoglutarate by mung bean mitochondria in the absence of adenylate. We decided to investigate more closely the effects of the supernatant fraction from avocado on avocado mitochondria. An increase in oxidation was observed with both fresh and deproteinized supernatant solution (table III). In some experiments the supernatant solution caused a slight increase in endogenous activity, probably by providing traces of metabolites. Whenever this occurred, the endogenous values were subtracted from the total rate of oxidation. It was observed in these experiments that the supernatant fraction had no effect on mitochondria extracted from hard fruit.

In order to relate the action of the supernatant solution to certain stages of ripening, fruit of known position in the climacteric curve were used for the preparation of cytoplasmic particles. The stage of ripeness was determined by following the respiration of the whole fruit and estimating its approximate stage on the climacteric curve. The increase in α-ketoglutarate oxidation due to deproteinized supernatant solution is shown in figure 2. Obviously the change in oxidative behavior of the particles and the effects of the supernatant factor are related to the climacteric rise. The striking response to DS prompted us to trace the function of the supernatant fraction in the metabolic reactions.

THE NATURE OF THE SUPERNATANT EFFECT: For all subsequent experiments a large quantity of supernatant solution was prepared from several ripe fruit following the procedure described under Methods. The solution was frozen in small aliquots which were thawed out as needed.

Assuming that the supernatant solution provided

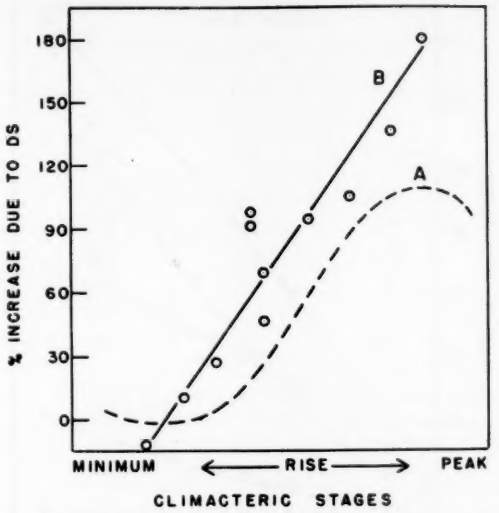

FIG. 2. Effect of the deproteinized supernatant fraction (DS) on the oxidation of α-ketoglutarate by particles extracted from fruit at different stages of the climacteric. A. The climacteric pattern in respiration of the intact fruit. B. Response to DS of particles extracted from fruit at different stages. Reaction mixture same as in table I.

some active metabolic factor, attention was now directed toward finding the locus of the activity in the Krebs cycle. Table IV shows experiments with pyruvate, citrate, α-ketoglutarate and succinate. The deproteinized supernatant solution stimulated the oxidation of α-ketoglutarate and pyruvate but not of succinate and citrate. The cofactor requirements for oxidation of Krebs cycle acids by avocado mitochondria were studied mostly on preclimacteric fruit. It was shown (4) that adenylate in any form was not required for succinate oxidation whether the preparation was washed once or twice. In the case of citrate the omission of AMP caused a 30 % reduction of the oxygen uptake in a highly washed preparation (1). Under the same conditions α-ketoglutarate oxidation was reduced by 60 %. The question arose, therefore,

TABLE IV

EFFECTS OF DEPROTEINIZED SUPERNATANT FRACTION (DS) ON OXIDATION OF KREBS CYCLE ACIDS BY MITOCHONDRIA OF RIPE AVOCADO

SUBSTRATE	Q_{O_2} (N)	
	CONTROL	DS
Pyruvate	0	82
α-Ketoglutarate	72	251
Citrate	191	180
Succinate	336	310

Reaction mixture: same as in table I, temp. 30° C, 0.5 ml DS where indicated.

TABLE V

COMPARISON OF THE EFFECTS OF ADENYLATE AND DEPROTEINIZED SUPERNATANT FRACTION (DS) ON THE OXIDATION OF α-KETOGLUTARATE BY PARTICLES OF RIPE AVOCADO FRUIT

CONDITIONS	Q_{O_2} (N)	% INCREASE DUE TO *DS*
Minus adenylate		
− DS	136	..
+ DS	202	48
Plus adenylate		
− DS	350	..
+ DS	615	76

Reaction mixture: same as in table I + 0.5 ml of DS where indicated.

whether the addition of DS increased the supply of adenylate to a level required for optimal oxidation of the keto acid. One might expect therefore a greater response from DS in the absence than in the presence of adenylate. Obviously this was not the case, as can be seen from table V. On the contrary, adenylate and the supernatant fraction acted synergistically. This observation is apparently in harmony with the findings of Millerd et al (13) that a heat stable component from the supernatant fraction of climacteric avocado brought about increased oxidation by mung bean mitochondria. However, they tested this component only in the absence of exogenous phosphate acceptor and conclude therefore that it "acts qualitatively like adenylate itself." From our results it seems more likely that DS interacts with rather than replaces adenylate.

The heat stable fraction of Millerd et al (13) caused a high percentage increase in phosphorus uptake though the actual magnitudes were low. In addition, a heat labile fraction acted like dinitrophenol in restoring the oxidation to the level of the complete system which contained adenylate. In this study a comparison of supernatant fraction with that of dinitrophenol (table VI) clearly indicates that neither exhibited uncoupling characteristics with respect to mitochondria from ripe avocados. This experiment is in conformity with the results given in table I and figure 1. In all cases active phosphorylation has taken place though the P/O ratios were lower than those reported previously with α-ketoglutarate. It is

TABLE VI

COMPARISON OF THE EFFECTS OF DEPROTEINIZED SUPERNATANT FRACTION (DS) AND DINITROPHENOL (DNP) ON OXIDATION AND PHOSPHORYLATION BY MITOCHONDRIA FROM RIPE AVOCADO

ADDITIONS	μATOMS O/HR	MICROMOLES P/HR	P/O
Control	8.6	6.0	0.7
DNP	7.8	6.0	0.8
DS	10.5	8.0	0.75

Reaction mixture: same as in table I; substrate, α-ketoglutarate; 0.5 ml of DS; 10^{-5} M DNP.

likely that the fruit used for the experiment given in table VI was in a more advanced stage of ripening than the material of table I, which was designated as fruit at the climacteric peak. The significant conclusion is that neither DS nor DNP reduced the P/O value.

In view of the fact that the keto acids responded most markedly to the supplementation of the reaction mixture with the supernatant fraction, it was deemed advisable to investigate additional cofactors. Coenzyme A activity has been studied most extensively in conjunction with pyruvate and α-ketoglutarate oxidation. The role of CoA in implementing the entry of acetate derived from pyruvate into the Krebs cycle was reviewed by Gunsalus (6). Hift et al (7) and Kaufman et al (9) demonstrated the importance of CoA in α-ketoglutarate oxidation. The requirement for CoA by avocado particles from unripe fruit was studied by Avron and Biale (1) for pyruvate oxidation only. It was plausible to assume that this factor was not dissociated readily from the mitochondrial complex of preclimacteric avocados. The role of CoA in particles of ripe fruit was investigated here in relation to the activity of the supernatant fraction. As demonstrated by experiments shown in table VII, CoA not only increased the oxidative rate in the absence of denatured supernatant, but resulted in an even greater increase when supernatant was present. As in the case with AMP, CoA and supernatant solution functioned in a synergistic manner.

The activity of CoA in the decarboxylation reactions involves LTPP as an essential component of the reaction mechanism (6, 16). This factor was added both in the presence and absence of supernatant, but it failed to show an effect in either case (table VII). The synergistic effect of CoA and the DS could be ascribed to the activation of CoA through a reduction. This was apparently not the case, since supernatant solution effects were obtained even in the presence of cysteine while cysteine itself did not increase the activity of CoA.

The synergistic responses in the above described

TABLE VII

EFFECTS OF COENZYME A (CoA) AND LIPOTHIAMINE-PYROPHOSPHATE (LTPP) ON α-KETOGLUTARATE OXIDATION IN RELATION TO THE EFFECT OF THE DEPROTEINIZED SUPERNATANT FRACTION (DS)

REACTION MIXTURE	Q_{O_2} (N)
Complete	120
Omit CoA	60
" DS	30
" CoA and DS	0
Complete	168
Omit LTPP	206
" DS	97
" DS and LTPP	99
" DS, LTPP and CoA	29

Reaction mixture: same as in table I; 0.5 ml DS where indicated; temp. 30° C.

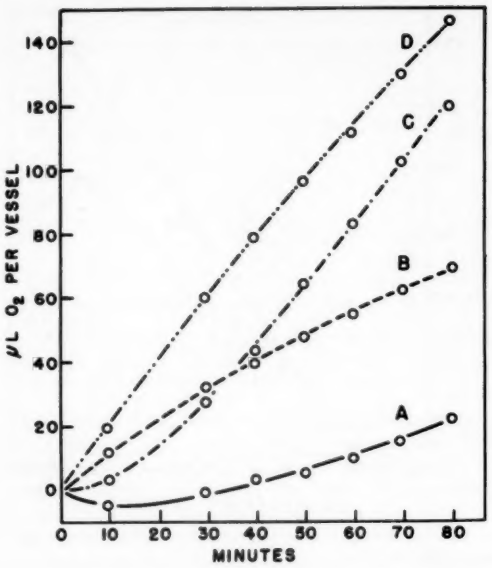

FIG. 3. The oxidation of α-ketoglutarate by cytoplasmic particles of the avocado fruit in relation to adenylate and the supernatant fraction (DS). A. AMP. B. ADP. C. AMP + DS. D. ADP + DS. Reaction mixture same as in table I.

experiments imply the function of the supernatant fraction in some phases of α-ketoglutarate oxidation in which all three factors, supernatant fraction, CoA and adenylate, are involved as intermediates. The form of adenylate would be of particular importance if the avocado mitochondria contain an α-ketoglutarate dehydrogenase system similar to that described by Hift et al (7) and Kaufman et al (9). These authors demonstrated a strict requirement for ADP as the phosphate acceptor. In oxidations by avocado mitochondria, AMP semed to function as an adequate phosphate acceptor (4). On the other hand, several experiments provided indirect evidence that ADP is in fact the essential adenylate. This contention is based on the lag period in oxidation (curves A and C, fig 3) observed with AMP but always absent (curves B and D) when ADP is used. Lindberg and Ernester (11) explained similar lag periods as the time required for the formation of enough ATP to provide ADP through the adenyl-kinase (myokinase) reaction:

$$AMP + ATP \rightleftharpoons 2\,ADP$$

Slater and Holton (17) observed a similar lag period in the phosphorylation of heart muscle sarcosomes. Their lag was also removed by ADP and they have shown an increased need for ADP with increasing dilution of the sarcosomes. A tabulation of our results in table VIII indicates that with avocado mitochondria as well, a longer lag period was obtained with decreasing mitochondrial nitrogen. As explained by Slater and Holton (17), the increased dilution dimin-

TABLE VIII

THE RELATIONSHIP OF THE LAG PERIOD TO MITOCHONDRIAL NITROGEN

	LAG PERIOD IN MINUTES		
	0–5	5–10	OVER 10
	mg N/0.5 ml suspension of particles		
	0.63	0.60	0.44
	0.76	0.40	0.50
	0.89	0.46	0.34
	0.72	...	0.33
	0.88	...	0.19
	0.73	...	...
Average	0.73	0.49	0.36

ishes the relative residual ADP supply requiring more time to form the necessary level for maximum oxidation.

DISCUSSION

The studies reported here indicate a decline in α-ketoglutarate oxidation during ripening and suggest that the α-ketoglutarate dehydrogenase might provide a desirable biochemical reaction for an analysis of the senescent drift in fruit respiration. This was supported by the effects of the DS and the correlation of this effect with the climacteric rise.

The ultimate aim of experimentation with the supernatant fraction is, no doubt, the determination of its intracellular function along with its purification and identification. With this long-term objective in mind, it was thought most expedient to determine the locus of activity in order to gain a lead to the probable function of the fraction as well as to its identity. Localizing the area of activity might indicate which enzymes should be purified to provide a sound assay system for further exploration. Our studies thus far have shown that the action of the supernatant fraction is centered in the substrate level reactions of α-ketoglutarate oxidation. This can be deduced from the localization of the effect to the α-keto acids, the synergistic activity with CoA and a similar synergism with adenylate. The significance of these synergisms is based on the assumption that avocado mitochondria contain an α-ketoglutarate dehydrogenase system similar to that described for animal mitochondria (7, 9). This assumption is supported not only by the requirements for similar cofactors, but also by the preference for ADP as the specific phosphate acceptor.

The presence of a supernatant fraction essential to the mitochondrial reactions could be explained by the existence of a state of equilibrium between the mitochondria and the surrounding medium. Under these conditions the factors essential to one reaction sequence could be lost while another reaction sequence remained unaffected. Such a theory was proposed by Potter et al (15) to account for a differential change in the enzymatic activity of rat liver mitochondria. This same reasoning could also apply to the avocado particles whose power to oxidize succinate remained stable while the ability to oxidize α-ketoglutarate declined during ripening of the fruit.

Dinitrophenol was used to enhance the rate of α-ketoglutarate oxidation. No enhancement was recorded in any stage of ripeness. DNP had no effect on oxidative activity whether it did or did not act as an uncoupling agent. Similar responses were obtained with malate, pyruvate, and succinate. The only definite effects of DNP were on phosphorylation and in particular on mitochondria from unripe fruit. In climacteric material the uncoupling effect was virtually eliminated. The operation of a DNP resistant phosphorylation is a unique phenomenon and is difficult to interpret until more is known about the mechanism of action of this substance. This phosphorylation could be ascribed to substrate level oxidation had it been limited to α-ketoglutarate. The data clearly indicate a DNP stable incorporation of phosphate with malate, succinate, and pyruvate as well as α-ketoglutarate. The fact that active esterification takes place during the climacteric rise suggests that ripening might require energy, an idea which is in harmony with Hulme's (8) findings on net protein synthesis in the apple, and with Tager and Biale's (18) report on the formation of aldolase and carboxylase in the banana. The question is therefore raised again whether ripening is a coupled or an uncoupled process. Our findings do not support the "Uncoupling" theory of Millerd, Bonner, and Biale (13). Neither do they contradict it directly. It should be emphasized that their ideas were based on the action of a heat labile factor, while most of our work was concerned with the heat stable fraction of the supernatant solution. Conceivably, uncoupling might be responsible for certain reactions associated with ripening but might not be the trigger mechanism. The application of DNP to intact fruit might throw some light on the role of uncoupling in ripening. Marks, Bernlohr, and Varner (12) injected DNP into green mature tomatoes and reported that this material remained unchanged in appearance from freshly harvested product while the control ripened normally. Thus far no evidence of this kind is available for the avocado. If energetic coupling is a prerequisite for the ripening process it will be necessary to postulate new ideas on the mechanism of the induction of the climateric rise. The revised orientation might include changes in metabolic pathways similar to those reported by Tager and Biale (18).

SUMMARY

The oxidative and phosphorylative activities of cytoplasmic particles isolated from the avocado fruit were studied in relation to the process of ripening and senescence.

The oxidation rates of α-ketoglutarate and malate were markedly reduced at the climacteric peak, while the P/O ratios tended to be high. The presence of dinitrophenol had no effect on the phosphorylative capacity of the particles at the peak, though it did lower the P/O ratios at the earlier stages of ripening.

The loss of oxidative activity of the α-ketoglutarate and pyruvate systems could be restored substantially by the addition of deproteinized supernatant fraction from ripe fruit. This solution exerted no influence on citrate and succinate oxidations, but with α-ketoglutarate its action was directly related to the stage of ripeness along the climacteric curve.

The idea was advanced that the role of the supernatant fraction was centered around the substrate level reactions of oxidative decarboxylation. This contention was based on the localization of the effect of the α-keto acids and on the synergistic activities with CoA and adenylates.

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BIOCHEMICAL CHANGES DURING GERMINATION OF THE TUNG SEED[1]

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Air-dry tung seeds usually germinate slowly and unevenly. Sharpe and Merrill (6) improved both the rate and uniformity of germination by stratification of the hulled seed outdoors in sand about 2 months prior to planting. Shear and Crane (7) also accelerated germination of tung seeds by soaking them in aqueous solutions containing morpholine (a heterocyclic ring compound derived from ethanolamine, which was used to reduce surface tension in water). In the germination of dry-stored tung seeds, Johnston and Sell (4) found that lipase activity and the utilization of the reserves occurred after the emergence of the radicle from the seeds. Similar observations on the transformation of substances in tung seeds have been reported also by Gerschtein (3). The present study was made to correlate utilization and translocation of reserves to the seedling and respiratory activity with rate of emergence and early stages of germination.

[1] Received revised manuscript July 18, 1957.

METHODS

In November 1942, about 9000 seeds were obtained from a single tree (F-56). On December 22, 1942, a random lot of 2200 seeds was placed on top of 2 inches of sand in a 4×2-foot outdoor stratification bed and then covered with 10 inches of sand. The remaining seeds were kept in bags in an unheated shed until March 4, 1943, when they were subdivided into 2 lots of 3400 each. One lot designated as "dry-stored" was planted 3 inches deep in sand in an outdoor seedbed without further treatment. The second lot was treated with morpholine according to the method of Shear and Crane (7) and then planted in the sand. The seeds previously stratified also were planted on the same date. Samples ranging from 150 seeds in the beginning to about 50 seedlings in the last 2 stages of germination were collected from all treatments at 9 times as follows:

(1) December 22, 1942, at the initiation of the experiment.
(2) February 5, 1943, after 45 days of stratification or dry storage.
(3) March 5, 1943, at planting time.
(4 to 7) At stages of growth classified according to the length of radicle as the 3-, 40-, 65-, and 100-mm root stages.
(8) Root 150 mm long, endosperm hard, hypocotylar arch developed.
(9) Root 150 mm long, endosperm soft.

In this experiment, development of the seedlings was studied for a longer period than in that of Johnson and Sell (4) in which the last sample was taken at the 65-mm root stage.

The apparatus devised by Ulrich (8) for measurement of root respiration was adapted for these studies by using moist absorbent paper in the respiration chamber (4-liter bottle) instead of nutrient solution. All samples consisted of seeds with the shells removed (hereafter designated "kernel"). In the early stages, about 100 to 150 kernels were used to give about 10 ml (S T P) of gas exchange in a 6-hour determination. Since the oxygen-producing capacity of the apparatus was about 200 ml per hour, only 20 to 50 seedlings could be used in the late stages, and the period had to be shortened to 2 or 3 hours.

The methods were essentially those previously described (4) except for the following modifications. A preliminary drying of all samples was conducted in vacuo at room temperature over P_2O_5 until about 90 % of the moisture had been removed. The drying was then continued at 40° C until renewed desiccant showed no deliquescence; subsequently, the temperature was raised to 70° C for drying to constant weight. All oil extractions were first made on flaked kernels. After being ground to 60 mesh in an intermediate Wiley mill, the residue was re-extracted. Correction was made for loss of material in grinding.

Amylase was determined by the procedure of Bernstein (1). About 1 gm of ether extracted meal was ground in 100 ml of water with sand in a mortar and the centrifuged supernatant used for the amylase assay (40° C for 2 hours). The reducing sugar formed was estimated by a modification of the Munsen-Walker method (2). A Taka-diastase preparation (Parke-Davis & Co.) was used as a standard, the activity being expressed in terms of micrograms of Taka-diastase. The units of activity are, therefore, only relative.

RESULTS

The seedlings from stratified seeds developed very uniformly, and by March 18 (13 days after planting) most of them had reached the 3-mm stage (table I). By April 11, 37 days after planting, the final (soft endosperm) stage was attained. The morpholine-treated seeds were next to germinate; the emergence of radicles was noted first on March 23, and the majority of the seedlings were at the 3-mm stage when sampled on March 29. The 150-mm stage was attained by April 12. However, most of the seedlings from morpholine-treated seeds were killed by a rot. There was practically no infection in the adjacent sections of the bed containing seeds given the other treatments; therefore, the morpholine treatment may have predisposed the seeds to the disease. Since only partial data were obtained for the morpholine-treated seeds, only the results obtained with dry-stored and stratified seeds will be presented. The dry-stored seeds began germinating about March 29, and the first stage was sampled by April 14. The final stage was attained on April 30.

The treatments produced outstanding differences in uniformity as well as rate of germination. At each sampling date, at least 50 % of the stratified seeds were in the same growth stage in contrast to only about 25 % of the dry-stored seeds.

Air-dried seeds had a very low gas exchange of less than 0.1 ml per hour per 100 kernels. A supplementary experiment showed that on soaking the seeds there was about a ten-fold increase in respiration, and during the first 12 hours, there was an excess of CO_2 evolved over O_2 absorbed. Within 34 hours after water absorption began, the RQ had fallen to 1.0. The RQ value of 10 given for dry-stored seeds in table I, therefore, represents only the condition prior to water absorption. It is presumed that these values would have reached unity within approximately 48 hours after planting in moist sand.

The stratified seeds had attained RQ values close to unity during stratification. Supplementary determinations made during the period between planting and the emergence of the radicle gave values of about 0.7. By the time of visible germination (3-mm stage), the RQ of stratified seeds had reached a relatively constant minimum, about 0.4. At the 150-mm hard endosperm stage, it was still low but rose when the endosperm softened.

At the 3-mm stage, the endosperm of dry-stored seeds produced more CO_2 but absorbed less O_2 than that of the stratified seeds with a resultant RQ of 0.7. In dry-stored seeds, the low RQ characteristic of oil

TABLE I

CHANGES DURING GERMINATION IN RESPIRATORY EXCHANGE OF DRY-STORED (D) AND STRATIFIED (S) TUNG SEEDS AS RELATED TO DRY WEIGHT AND MOISTURE CONTENTS OF ENDOSPERM AND EMBRYO

GROWTH STAGE *	CO_2 ML/HR × 100 KERNELS **		O_2 ML/HR × 100 KERNELS		RQ †		DRY MATTER: GM PER ENDOSPERM		DRY MATTER: GM PER EMBRYO		MOISTURE: GM PER ENDOSPERM		MOISTURE: GM PER EMBRYO	
	D	S	D	S	D	S	D	S	D	S	D	S	D	S
Dormant	0.02	....	0	...	20	..	2.3	...	43	....	0.2	..	3	....
Preplanting	0.01	5.1	0	5	10	1.1	2.4	2.3	50	50	0.2	0.9	3	26
Planting	0.10	11.8	0	14	10	1.1	2.3	2.3	44	53	0.1	0.8	11	29
Root														
3 mm	32.1	28.1	45	66	0.7	0.4	2.5	2.5	62	67	1.2	1.0	79	72
40 mm	84.8	56.3	164	123	0.4	0.5	2.4	2.3	105	103	1.5	1.7	299	278
65 mm	104.0	139.3	183	310	0.5	0.4	2.3	2.3	348	235	3.1	2.5	1052	752
100 mm	261.1	158.0	535	332	0.5	0.5	2.1	2.1	660	788	4.2	4.2	2223	1910
150 mm														
Hard endosperm	215.2	315.2	424	712	0.5	0.5	1.2	1.8	1280	985	6.5	4.8	2915	2536
Soft endosperm	127.8	334.4	122	437	1.0	0.8	1.1	0.8	1800	2025	6.3	7.4	4720	4701

* Dormant, December 22; preplanting, February 5; planting, March 5.
** Seeds with shells removed.
† Ratios of gases for each run, not necessarily same as ratio of gas vol.

utilization occurred first at the 40-mm stage. The RQ values remained low, although slightly higher than corresponding values for stratified seeds, through the next three stages but rose at the final stage. The generally accelerated respiration rate characteristic of developing seedlings was observed, the peak for the stratified seeds occurring when the roots were about 150 mm long, and there was considerable hypocotyl elongation. The peak for dry-stored seeds occurred at the preceding stage.

Distinct decreases in oil content of the endosperm occurred at the 40-mm stage of stratified seeds and the 65-mm stage of dry stored seeds (table II). In the corresponding embryos, oil attained a maximum at the 100-mm and the 65-mm stage, respectively, for the stratified and dry-stored seeds and then declined rapidly.

Free fatty acids were present in both endosperm and embryo of dry-stored seeds as early as the 3-mm stage, and there was a distinct increase in quantity at all the subsequent stages. No free fatty acid was found in either endosperm or embryo of stratified

TABLE II

CHANGES DURING GERMINATION IN OIL CONTENT AND QUALITY OF OIL OF ENDOSPERM AND EMBRYO OF DRY-STORED (D) AND STRATIFIED (S) TUNG SEEDS

GROWTH STAGE *	ENDOSPERM FRACTIONS: OIL GM/SEED		IODINE NO.		SAPON. NO.		ACID NO.		EMBRYO FRACTIONS: OIL GM/SEED		IODINE NO.		SAPON. NO.		ACID NO.	
	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S
Dormant	1.5	..	159	...	192	...	0	..	25	..	143	...	192	...	0	..
Preplanting	1.5	1.5	159	160	195	196	0	0	29	30	135	138	195	194	0	0
Planting	1.4	1.5	158	161	194	192	0	0	27	31	140	144	194	192	0	0
Root																
3 mm	1.6	1.6	162	163	191	196	1	0	31	34	142	138	191	192	7	0
40 mm	1.5	1.4	162	163	190	194	7	0	32	27	142	142	197	195	13	0
65 mm	1.1	1.2	163	159	195	196	7	0	44	28	130	136	220	197	27	0
100 mm	0.6	0.8	148	161	215	191	19	0	16	52	125	126	221	202	22	0
150 mm																
Hard endosperm	0.3	0.6	138	156	195	192	37	5	3	15	...	125	...	202	..	31
Soft endosperm	0.2	0.1	142	150	189	192	58	36	8	13	...	119	...	181	..	44

* Same at table I.

seeds until the emergence of the hypocotyl at the 150-mm, hard-endosperm stage.

The iodine numbers and refractive indices (data not given) of the oils from the two fractions of dry-stored seeds remained essentially constant through the first two or three stages of root elongation and then decreased. Corresponding declines in the case of stratified seeds were found only in very last (soft-endosperm) stage.

The embryo oil of dormant seeds differs qualitatively from the endosperm oil in having a higher degree of saturation of the fatty acids of the glycerides than endosperm oil, which forms the bulk of commercial tung oil. These differences are indicated by lower refractive indices (data not given) and iodine numbers of the embryo oil. However, there are no statistically significant differences in the saponification numbers of the oil of the two seed parts at the dormant stage or throughout the entire course of germination.

The data for carbohydrate and nitrogen fractions are given in table III for the endosperm of the seeds and seedlings and in table IV for the embryo portions. In both dry-stored and stratified seeds, there was a decrease in the nonreducing sugar (sucrose) in the seeds prior to planting. After emergence of the radicle, sucrose accumulated in the tissues of seeds given both treatments. The seedlings continued to increase in sucrose throughout the entire germination period, but sucrose decreased in the endosperm tissue after reaching a maximum at the 100-mm root stage. The initial decrease in sucrose content for the whole seed was noted in the previous study (4), but the subsequent increase was not observed since the older seedling stages in which it occurred were not sampled. Reducing sugars were absent from the embryo and were present only in low amounts in the endosperm at planting time. In both fractions, the amounts per seed increased steadily during germination and attained a somewhat higher maximum in the dry-stored than in the stratified seed. At the final stage, a decrease in reducing sugars occurred in the endosperm tissues.

Only traces of starch were present in the dormant seed, and the quantity remained low in the endosperm until the last stages of germination when a rapid accumulation occurred. Accumulation of starch occurred earlier in the embryo (65-mm stage), and the amount was much greater than in the endosperm. As was noted by Johnston and Sell (4), changes in other polysaccharides of the endosperm were without significance up to the 65-mm stage. These substances apparently decreased at the last sampling stages. The embryo growth, however, reflected the accumulation of other polysaccharides, which presumably are constituents of the cell wall material formed most rapidly in the late stages of germination.

Both alcohol-soluble and alcohol-insoluble nitrogen increased steadily in the embryo after radicle emergence. In the late stages of germination, alcohol-insoluble nitrogen decreased in the endosperm, presumably as a consequence of its translocation to the embryo. Alcohol-soluble nitrogen in the endosperm increased somewhat at the 40- to 100-mm stages and then decreased.

At the first visible signs of germination, lipase activity (table III) was slight in the dormant endosperm and increased in seeds given both treatments. This confirms the work of Gerschtein (3) and Johnston and Sell (4). The data in table III show

TABLE III

CHANGES DURING GERMINATION IN NITROGEN AND CARBOHYDRATE CONTENT AND ENZYMATIC ACTIVITIES OF ENDOSPERM OF DRY-STORED (D) AND STRATIFIED (S) TUNG SEEDS

GROWTH STAGE *	NITROGEN **				CARBOHYDRATES **								LIPASE ACTIVITY †		AMYLASE ACTIVITY ††	
	SOLUBLE		INSOLUBLE		REDUCING SUGARS		NONREDUCING SUGARS		STARCH		OTHER POLYSACCHARIDES					
	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S
Dormant	5	..	70	..	0	..	140	...	5	...	53	..	4	..	0	...
Preplanting	4	5	64	71	0	0	125	123	0	14	65	63	3	6	0	0
Planting	2	2	68	76	2	0	37	43	8	7	82	64	3	4	0	22
Root																
3 mm	4	6	67	78	1	9	43	40	2	3	66	65	6	16	0	...
40 mm	6	10	65	69	5	6	80	73	1	2	76	60	10	20	57	291
65 mm	9	7	52	64	29	7	192	105	6	6	82	67	31	18	114	524
100 mm	11	10	45	43	55	47	268	323	89	9	76	88	22	19	334	498
150 mm																
Hard endosperm	3	11	32	37	82	39	45	112	38	159	68	45	9	9	271	216
Soft endosperm	4	2	24	20	43	29	74	64	109	69	41	44	9	6	154	...

* Same as for table I.
** Mg per seed.
† Mg oil hydrolyzed per hr per seed.
†† Equivalent to activity of Taka-diastase in μgm per seed.

TABLE IV

CHANGES DURING GERMINATION IN NITROGEN AND CARBOHYDRATE CONTENTS AND ENZYMATIC ACTIVITIES OF EMBRYO OF DRY-STORED (D) AND STRATIFIED (S) TUNG SEEDS

GROWTH STAGE *	NITROGEN **				CARBOHYDRATES **								LIPASE ACTIVITY †		AMYLASE ACTIVITY ††	
	SOLUBLE		INSOLUBLE		REDUCING SUGARS		NONREDUCING SUGARS		STARCH		OTHER POLYSACCHARIDES					
	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S
Dormant	0.1	...	1.4	...	0	..	4	...	0	...	1	...	0.1	...	1	...
Preplanting	0.1	0.1	1.6	1.5	0	0	4	4	...	0	1	1	0.1	0.1	...	...
Planting	0.1	0.1	1.3	1.8	0	0	2	2	0	0	1	1	0.1	0.2	5	4‡
Root																
3 mm	0.1	0.4	1.5	2.3	1	0	4	5	1	0	2	1	0.3	0.5	19	37‡
40 mm	1.0	0.8	4.2	4.4	4	4	11	14	6	7	3	3	0.7	1.4	81	161‡
65 mm	3.5	1.4	8.4	6.4	9	6	50	25	62	45	14	7	1.5	2.5	339	143
100 mm	4.9	4.3	17.5	20.5	12	16	69	98	185	210	31	38	4.7	10.1	349	433
150 mm																
Hard endosperm	8.4	11.3	26.9	18.4	32	37	140	172	415	297	65	57	3.9	7.1	561	434
Soft endosperm	16.1	20.8	33.8	37.4	67	59	195	252	563	638	..	135	2.0	10.5	602	467

* Same as for table I.
** Mg per seed.
† Mg oil hydrolyzed per hr per seed.
†† Equivalent to activity of Taka-diastase in μgm per seed.
‡ Data from morpholine-treated seeds.

greater lipase activity at the early stages of germination in the endosperm of stratified seeds than in that of the dry-stored. The marked increase in lipase activity in the endosperm of the dry-stored seed at the 65-mm stage was associated with a relatively marked decrease in oil content. In the final stages of germination, a decrease in lipase activity was found in the endosperm of seeds given both treatments.

The embryo fraction showed comparatively little lipase activity until about the 100-mm stage. By this stage, the lipase activity in the embryo of the stratified seeds was about one-third of the total of the whole seed. In dry-stored seeds at this stage, the embryo contained about one-sixth of the total.

Since insufficient material was available for amylase determinations on certain embryo samples, the study of amylase activity was incomplete. The data in tables III and IV show practically no amylase activity in the dormant seed. There was a great increase in amylase in both endosperm and embryo concomitant with embryo growth. The increase in amylase preceded the increase in starch, particularly in the endosperm. Supplementary determinations, not included in these tables, showed that the cotyledons at the last seedling stage were particularly rich in amylase. As in the case of lipase, the stratified seed showed greater total amylase activity than the dry-stored during the stages from the emergence of radicle to those just prior to softening of the endosperm.

DISCUSSION

The data presented indicate that the germination process in tung seeds follows a characteristic course for oily seeds with certain modifications produced by seed treatment prior to planting.

The earlier and more uniform initiation of germination in the stratified seeds may be attributed to a separation in time of the two phases of the germination process: the imbibition of water and the utilization of the food reserves. The temperatures of the stratification bed remained uniformly low at about 18° C during the two months of treatment. Comparatively small differences in water absorption by tung seeds are found over a range of storage temperatures from 7 to 35° C (unpublished data). Respiration and growth are influenced much more by temperature. In this work, as much as a two-fold difference in gas exchange in a water bath at 25° C was noted between lots of germinating seed obtained in the morning when the seedbed was cold (15 to 18° C) and corresponding lots obtained in the afternoon when the seedbed was warm (25 to 30° C).

At the low temperature of stratification, most of the seeds had time to absorb enough water for germination, but respiratory activity remained low. Thus, the effects of differences between individual seeds in rate of water absorption were minimized. On transfer to the higher temperatures of the seedbed, the hydrated seeds sprouted readily and produced a highly uniform stand.

When seeds were planted dry in the germination bed, both the necessary water and temperature for germination were supplied simultaneously, and the rate of water absorption was a major limiting factor. Thus, differences in water uptake by individual seeds were reflected in the variability of the stand. Mor-

pholine treatment tended to eliminate this water-uptake factor and helped to produce a better stand until the rot developed.

Although the moisture in the stratified seeds had already become about 40 % of the total weight at the time of planting, the increase of respiratory activity coincident with moisture absorption of dry-stored seed is, therefore, not necessarily a consequence of the hydration of the seed after planting. Since the seeds were shelled for respiration measurements, permeability of the seed coat to oxygen was not a factor in these experiments.

Necessary physiological processes other than water absorption take place during stratification. Merrill (5) showed that even when morpholine-treated and stratified seeds of an approximately equal moisture content (40 %) were planted in March under identical growing conditions, the stratified seed germinated in 10 days compared with 21 days for the morpholine-treated.

These biochemical changes during stratification were evidenced in several ways. The enzyme activities in stratified seed after planting were higher than those of dry-stored seed; the RQ value dropped more rapidly; and the nature of the changes in oil characteristics also indicated more effective enzyme systems. The absence of free fatty acids in the oil of stratified seed and the relatively constant saponification numbers showed that utilization of the hydrolyzed oil was proceeding at a greater rate than was the lipolytic reaction.

The greater degree of unsaturation characteristic of the oils of most of the stratified seed tissues was also indicative of more efficient utilization since an addition to the double bonds must take place during utilization as free fatty acids. An overall reaction by which eleostearic acid could be oxidized to carbohydrate and have an RQ of 0.48 is given by the following equation:

$$2\,C_{18}H_{30}O_2 + 25\,O_2 \longrightarrow 4\,C_6H_{12}O_6 + 6\,H_2O + 12\,CO_2$$

$$RQ\ \frac{12\ CO_2}{25\ O_2} = 0.48$$

The existence of such an overall reaction is strongly suggested by the average RQ of 0.49 for all except the initial and final planted seeds.

The formation of insoluble starch grains is an end product of this reaction, which tends to be reversed by the accumulation of glucose. The higher amylase and lower reducing sugar contents of the stratified endosperm during the early stages of germination in contrast to those of dry-stored endosperm are probably related to more rapid utilization during growth.

It is evident, therefore, that a major effect of the preplanting treatment is on the relative rate of the processes involved in utilization of the oil stored in the seeds as a reserve food. The earlier initiation, greater activity and greater efficiency of the necessary enzyme systems which result from the stratification treatment must account for a considerable portion of the beneficial effects of stratification on rapidity of germination.

Starch synthesis was preceded by an increase in amylase activity. Again, the probable intermediate of the process, reducing sugar, was less evident in the early stages of germination of the stratified than in those of the dry-stored seeds.

Thus, the major differences in the metabolism of germination of the seeds under these treatments are those produced by a more rapid and more effective utilization of reserves in the stages of germination preceding rapid growth of the hypocotyl. The less highly developed enzymatic system of the dry-stored seeds results in an accumulation of intermediates with some factor associated with growth rate being limiting.

Summary

The changes in chemical composition, respiratory gas exchange, and enzymatic activities were followed through the germination of dry-stored and stratified tung seeds. The stratified seeds germinated more rapidly and uniformly than did the dry seeds. This effect of treatment was associated with the earlier absorption of water and activation of the enzymes, lipase and amylase in the stratified seeds. The higher enzyme activity in turn was reflected in a more efficient utilization of oil reserves as indicated by a more rapid drop in RQ values following planting and by the absence of free fatty acids in either the endosperm or the embryo oil of the stratified seeds until the seedlings had reached the stage preceding dissolution of the endosperm. As soon as the dry-stored seeds sprouted, free fatty acids were found, and the quantity increased rapidly as germination proceeded. The formation of saturated intermediates during the process of oil utilization was evident earlier in the oils of dry-stored seeds than in those of the more efficient stratified seeds. One end product of oil utilization was starch, which increased rapidly in the developing seedling while the oil of the endosperm decreased at a corresponding rate.

At all stages of germination, irrespective of treatment, the embryo oil had a lower percentage of unsaturated glycerides than the endosperm oil. This indicates that the fatty acid radicals remain essentially unchanged in molecular weight prior to utilization with no detectable accumulation of lower molecular weight intermediates during their utilization.

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CATALASE AND CHLOROPHYLL DEPRESSION BY 3-AMINO-1,2,4-TRIAZOLE[1,2]

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In a previous report (4), concerned primarily with the effect of 3-amino-1,2,4-triazole (AT) on animal catalase, some apparent anomalies were indicated in the comparative response of catalase activity and chlorophyll content of different plants treated with AT. Experiments reported in this paper are aimed at resolving some of these questions and throwing some light on the manner of action of AT on plants.

We had observed that when potato plants are grown from tubers in the soil, and the leaves sprayed lightly with 10^{-3} M AT, the new leaves which develop, subsequently, will vary from mottled to almost completely colorless. The catalase activity of these leaves does not differ from that of the controls sprayed with water alone. On the other hand, when nine-day-old barley seedlings are transferred to nutrient solution containing 10^{-4} M AT, the catalase activity of the leaves already developed and of those that follow is reduced by 50 % or more. The chlorophyll of the part of the leaves already developed is unaffected while the developing base of the leaf is yellow to white. Microscopic examination of the chlorotic tissue of both barley and potato leaves reveals that the plastids are few in number, shrunken and misshapen.

For these reasons, we postulated that although AT may have a more or less direct effect on catalase, the effect on chlorophyll was the result of the suppression of the normal development of the chloroplasts. AT is concentrated at the growing points, the shoot and root tips and, in barley, also the leaf meristem at the base of the leaf. Small amounts of AT had apparently been concentrated at the shoot tips of the potato plants causing the leaves that developed afterwards to be partially devoid of chlorophyll and the other plastid pigments. By the time the chlorotic leaves were harvested, the small amount of AT had either moved on with the growing point, been inactivated or had been diluted, and the catalase was back to normal.

Three groups of experiments were undertaken to test this hypothesis. One sought to effect a reduction of potato leaf catalase in vivo with AT. The second sought to demonstrate that the lower or younger part of the barley leaf was more affected by the AT than the upper or older part of the leaf—the former being closer to the AT concentration at the leaf meristem. The third series of experiments aimed at showing a more direct effect of AT on plant catalase by incubating low concentration AT with a barley leaf suspension. Methods for determining catalase activity and chlorophyll have been described previously (1, 2).

Uniform cuttings were taken from russet potato plants and rooted in vermiculite under mist. The rooted cuttings were transferred to aerated crocks containing a complete mineral nutrient solution. A week later, when the plants were growing vigorously and the roots were plentiful in the medium, the mineral solutions were changed and to part of the crocks AT was added in quantity to make the solution 10^{-5} M. Chlorotic leaves, which had grown out since the addition of AT, were harvested ten days later along with equivalent leaves from the controls. Catalase activity and chlorophyll content were determined on these leaves, and it was found that chlorophyll was reduced to 3 % of normal as expected and also that catalase activity was reduced to 44 % of normal (table I). It was also noted that root growth had stopped with the addition of AT. Further evidence for the ability of AT to reduce the catalase activity of the potato in vivo was obtained by allowing mature potato leaves to stand with their petioles in 10^{-3} M AT for 48 hours. Catalase activity was found to be 23 % of that of similar leaves kept in tap water.

Table II shows the effect of adding 10^{-4} M AT to the nutrient solution of 13-day-old barley seedlings grown on screens in 400-ml beakers (3). Plants were harvested 3 days after AT was added. We do not

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TABLE I

CATALASE AND CHLOROPHYLL IN POTATO LEAVES TREATED WITH AT

TREATMENT	DAYS AFTER TREATMENT	CATALASE		CHLOROPHYLL	
		UNITS*/GM FRESH WT	% OF CONTROL	MG/GM FRESH WT	% OF CONTROL
Developed after spraying plant with 10^{-3} M AT	8	2.8	104	0.024	1.5
Control	8	2.7	...	1.730	...
Developed on rooted cuttings—roots in 10^{-5} M AT	10	1.3	44	0.067	3.0
Control	10	2.8	...	2.000	...
Mature leaves—petioles in 10^{-3} M AT	2	0.7	23	No apparent difference	
Controls in tap water	2	2.9	...		

* The catalase unit is defined as the amount of catalase required to liberate one ml of O_2 from H_2O_2 at 0° C under our experimental conditions.

consider the differences indicated between the first and second leaves to be significant. We had thought that the relative maturity of the first leaf would cause the AT to be concentrated in the more rapidly growing second leaf, however the meristem of the former must still remain sufficiently active to bring AT to the growing point from which it diffuses upward. Note that in the lower half of the leaf the catalase activity is reduced to 23 % of that of the controls, while the upper half is reduced only 46 %. In these barley experiments no tissue emerging after the addition of AT was used, and so no chlorophyll depression was expected. As noted above, the emerging tissue is almost completely colorless. It is also so weak that the leaves bend over soon after this tissue appears thus making further work with those plants impossible.

To demonstrate in vitro inactivation of catalase by AT, normal barley leaves were ground in cold 0.1 M K_2HPO_4, strained through cheesecloth and diluted to 50 mg fresh weight per ml of suspension. This suspension was divided into two parts; into one, AT was added to make 10^{-3} M. Both fractions were allowed to stand at room temperature for several hours. The first catalase determinations were made after two hours, and at that time the activity of the AT fraction was 50 % of that of the normal fraction. Subsequent determinations showed that the activity of both suspensions was declining, but the AT always dropped faster than the normal, and at no time did the AT suspension rise above 50 % of the control. Since our earlier attempts at in vitro inhibition had been unsuccessful unless higher concentrations (0.1 M) were used, parallel suspensions similar to those described above were set up and stored for 96 hours at 4° C after which time they were removed to room temperature for 24 hours. After 96 hours in the cold the catalase activity was reduced only to 90 % of normal, while 24 hours more in the room temperature (23 to 25° C) reduced it to 60 % of controls.

TABLE II

CATALASE AND CHLOROPHYLL IN BARLEY LEAVES HARVESTED 3 DAYS AFTER 10^{-4} M AT WAS ADDED TO NUTRIENT SOLUTION OF 13-DAY-OLD SEEDLINGS

SAMPLE		CATALASE		CHLOROPHYLL	
		UNITS*/GM FRESH WT	% OF CONTROL	MG/GM FRESH WT	% OF CONTROL
First leaf	AT	0.64	55	0.75	92
	Control	1.15	...	0.82	...
Second leaf	AT	0.52	41	0.72	100
	Control	1.27	...	0.72	...
First leaf					
Upper half	AT	0.79	46	...	...
	Control	1.71	...	...	...
Lower half	AT	0.27	23	...	...
	Control	0.93	...	...	...

* The catalase unit is defined as the amount of catalase required to liberate one ml of O_2 from H_2O_2 at 0° C under our experimental conditions.

Apparently the catalase of macerates is sensitive to AT inactivation, but the inhibition is slowed down drastically by low temperatures. Similarly, we are forced to conclude that the reports of no catalase activity depression in potato in the presence of AT are in error, for it now seems likely that the AT was no longer present in those tissues. On the other hand, where we can be reasonably certain that AT is present the reduction of catalase activity always occurs. With chlorophyll the situation is different since a small amount of AT interferes with the plastids during leaf formation or differentiation, and chlorophyll as well as the other plastid pigments are always low in these leaves. With cessation of treatment a new flush of growth may appear which is now completely normal. Just what has happened to the AT, we are not prepared to say at this time; it may have just been diluted, or it may have been metabolized or inactivated in some other way.

SUMMARY

Further work on the effect of low concentrations of 3-amino-1,2,4-triazole on plants shows that catalase

activity is depressed whenever AT is present in the tissue. Low concentrations of AT have no effect on the chlorophyll content of mature leaves, but through interference with the developing plastids, a permanent reduction of the leaf's ability to manufacture chlorophyll occurs when AT is present at the time of differentiation, even though a subsequent disappearance of the AT causes the catalase to return to normal. Temperature sensitive in vitro inhibition of catalase activity with 10^{-3} M AT has been accomplished.

We are indebted to the American Cyanamid Company, Agricultural Chemicals Division, for the 3-amino-1,2,4-triazole.

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EDITOR'S PAGE

With this issue of *Plant Physiology* my duties as editor are over. Since September of 1957, Professor Allan H. Brown, the new editor, has been actively engaged in selecting manuscripts for volume 33, and I turn the journal over to him with confidence that it is in good hands. I would, however, pause to thank all who have aided me in the 4.5 years of the editorship. The members of the Editorial Board have been generous with their aid. A very large number of reviewers have contributed greatly to raising the quality of the published papers.

To our numerous authors, I wish to send my particular thanks; they have been appreciative of the suggestions of the reviewers and editor. Even more, they have been tolerant when my suggestions were without merit and generous in forgiving my mistakes. I had been warned by an experienced editor, "never to express a personal opinion, but to always hide behind the reviewers and the editorial board." Such advice was not suited to my temperament. Fortunately, our authors have seemed to appreciate direct criticism, and I have had as a result a vigorous and pleasant correspondence. The quality of our journal, in the last analysis, depends upon our authors, and they have shown that they welcome any criticism, however searching, that improves the quality and clarity of their papers.

Perhaps a retiring editor may be permitted to express some general views, at a time when his opinions will no longer determine the direction of the journal.

Scientific discovery becomes a part of the general body of knowledge by publication. The purpose of publication is to communicate the results of scientific observation and experiment throughout the world and to preserve that information for the future. Though other methods of communication exist, no other is so precise, so generally available, and so permanent as publication in an established journal of wide circulation. Though unpublished lectures and papers read at scientific meetings serve a purpose, they are no substitute for publication.

If our authors will recognize the nature of scientific communication, they can perhaps consider their own manuscripts more objectively. In writing their papers, in designing tables and graphs, the purpose should be to communicate the results of their studies and their conclusions in an unambiguous form, and in language that can be understood not only in the current year in these United States, but by physiologists of all countries and for future times. Some authors have objected to the editor's insistence on correct English and his objection to laboratory slang. The non-American reader (and they constitute nearly 50 % of our subscribers) may be unable to find in an unabridged dictionary the laboratory jargon in submitted manuscripts. If the results are worthy of publication, they are worthy of the effort required to obtain clarity and precision. Effectively used, the English language is a powerful tool in scientific communication.

Though a well-written scientific paper should be brief, the exaggeration of brevity has led to telegraphic reporting without precision or grace.

The objective style of scientific writing has many virtues. It avoids subjectivity and results in unemotional and unexaggerated reporting. These desirable features, however, should not require a stilted language or inverted word order. Though an impersonal tone is usually desirable, there is no need to suppress entirely the personal element in scientific papers. Indeed the studied avoidance of the pronoun "I" frequently leads to clumsy writing.

Of even more concern is the suppression of all the aesthetic aspects of scientific research. The best of research involves creative activity of high order, and the scientist has pleasure in the design of his experiments and particularly in the unexpected discovery or the experimental confirmation of his intuition. The deliberate suppression in scientific writing of the joy of scientific discovery and the aesthetic aspects of science, impoverishes the literature as well as the life of science.

The author who relates his report to previous knowledge in the same or other fields, increases the interest and value of his paper. This does not require a long introduction nor innumerable references. I, at least, find pleasure in learning from an article in *Plant Physiology* that John Evelyn's *Sylva* published in 1664, is pertinent to a modern problem.

When and what to publish, is the major problem; and not a major problem for the editor alone but primarily for the author. Not every observation is a scientific discovery; not every experiment is worth reporting. The number of possible observations and experiments is so great, and many are so relatively meaningless, that such observations should be published only if they make significant additions to the body of scientific knowledge. However, other verified observations may appear to be so in conflict with established theories, that their publication is required. Some relatively isolated observations may be so timely, and essential to other workers, that prompt publication in brief form is imperative. However, one cannot long serve as editor without being impressed by the large number of preliminary reports and series of short papers.

Frequently, the purposes of scientific communication would be better served by one reasonably complete paper than by several short ones. If I may repeat, the reviewers and the editor must evaluate each manuscript as a scientific communication. They are not judges of academic promotion, nor reviewing boards for the renewal of research grants. (I firmly believe that one major contribution will do more for the author's advancement than a series of partial reports.) I am reminded of a story of Jacques Loeb meeting a plant physiologist at Woods Hole and calling to him, "What is the meaning of the series of

telegrams you have been publishing—each new one contradicts the preceding one." Perhaps your retiring editor is only a 20th century Don Quixote tilting at a deluge of papers.

It is a pleasure to acknowledge my debt of gratitude to the two previous editors of this journal. Professor Charles A. Shull undertook the founding of this journal in 1925, bringing out the first issue in January 1926. He served continuously as editor through 1945. Walter E. Loehwing, his successor, served as editor from 1946 through the July issue of 1953, when the present retiring editor took over. I appreciate the honor the Society has shown in entrusting the journal to me, and I hope Professor Brown will have as much satisfaction in his period of editorship as I have had.—DAVID R. GODDARD.

ERRATA

Volume 31:

Page 459, column 1, 2nd line from bottom, *callected* should read *collected*.

Page 460, table IV, columns 4 and 7, *millimoles/hr* should read *meq/hr*.

Page 461, column 1, paragraph 1, line 8, *1953-A* should read *1952-A*.

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